In re Application of: Sergei BRAUN
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Examiner: Gennadiy MESH
Group Art Unit: 1796
Attorney Docket: 29948

REMARKS

Reconsideration of the above-identified application in view of the amendments above and the remarks following is respectfully requested.

Claims 114-139, 141-144 and 146-153 are currently pending in this Application. Claims 114-131 and 145-153 have been withdrawn from consideration as being drawn to non-elected invention. Claims 132-139 and 141-144 have been examined on the merits. Claims 1-113, 140 and 144 have been canceled in a previous response.

Claims 132-139 and 141-143 have been rejected under 35 U.S.C. §103.

35 U.S.C. §103(a) rejections

The Examiner has stated that claims 132-139 and 141-143 are rejected under 35 U.S.C. §103(a) as being unpatentable over Goodman et al. in view of Gouesnard. The Examiner's rejection is respectfully traversed.

Specifically, the Examiner has stated that Goodman et al. discloses hydrolysable copolymers of natural amino acids (reading on radical B) and hydroxyacids linked by an ester bond. The Examiner has further stated that Goodman et al. is silent regarding the use of specific radicals A, but that Gouesnard teaches hydroxy amino acids. The Examiner has therefore concluded that it would have been obvious to one of ordinary skill in the art to use hydroxy amino acids taught by Gouesnard instead of hydroxyacids in order to prepare hydrolysable polyesters as disclosed by Goodman et al. with reasonable expectation of success.

Applicant wishes to note that claims 135-139 and 141-143 recite a radical B that is clearly different than the amino acid residues taught by Goodman et al.

Hence, Applicant believes that the Examiner has failed to establish a *prima facie* case for the obviousness of the subject matter of claims 135-139 and 141-143.

In addition, Applicant believes that the Examiner has failed to make clear the rationale behind the conclusion that it would have been obvious to modify the hydrolysable polyesters taught by Goodman et al. by replacing the hydroxyacids taught therein with the hydroxy amino acids taught by Gouesnard. The Examiner

merely states that it would be obvious to one of ordinary skill in the art, and that there is a reasonable expectation of success. Moreover, as it is unclear what motivation would lead one of ordinary skill in the art to modify the teachings of Goodman et al. in such a manner, it is unclear what would constitute "success".

As discussed in MPEP §2143, the key to a *prima facie* case of obviousness is the clear articulation of the reason(s) why the claimed invention would have been obvious. The Supreme Court in KSR noted that the analysis supporting a rejection under 35 U.S.C. 103 should be made explicit.

As further discussed in MPEP §2143, the rationale to support a conclusion that the claim would have been obvious is that "a person of ordinary skill in the art would have been motivated to combine the prior art to achieve the claimed invention and that there would have been a reasonable expectation of success." DyStar Textilfarben GmbH & Co. Deutschland KG v. C.H. Patrick Co., 464 F.3d 1356, 1360, 80 USPQ2d 1641, 1645 (Fed. Cir. 2006). If any of these findings cannot be made, then this rationale cannot be used to support a conclusion that the claim would have been obvious to one of ordinary skill in the art.

In view of the lack of a clear rationale why a person of ordinary skill in the art would have been motivated to combine the prior art, Applicant believes that the Examiner has failed to establish a *prima facie* case for the obviousness of the claimed invention.

Notwithstanding the above, Applicant contends that one of ordinary skill in the art would have no motivation to modify the teachings of Goodman et al. by introducing a hydroxy amino acid taught by Gouesnard, as suggested by the Examiner, for the following reasons:

a) the proposed modification introduces considerable technical difficulties for no apparent reason;

b) the proposed modification is contrary to the stated object of the invention of Goodman et al.

c) Goodman et al. teaches away from the proposed modification by specifically excluding the use of compounds such as hydroxy amino acids.

Applicant argued in the response to the Official Action mailed February 21, 2008, filed June 23, 2008, that the teachings of Goodman et al. can be achieved with a hydroxy amino acid only if the amino group in the hydroxy amino acid does not react to form an amide bond, and the hydroxy group therein does react to form an ester bond, and that Goodman et al. does not even remotely suggest how such a selective reactivity can be achieved. The use of a hydroxy amino acid instead of the hydroxyacids taught in Goodman et al. would create, for no apparent reason, the technical difficulty of having to prevent amide bond formation, in order to facilitate the desired ester bond formation.

The Examiner has stated that Applicant's arguments are not persuasive because some quantity of ester bond will always be present and that this is recognized by Goodman et al., that Goodman et al. recognized that different reaction conditions, specific solvent and specific catalyst will facilitate one type of reaction compared to another, that only a relatively small of ester bonds is necessary, and that Goodman et al. provides a method of isolating polymers with ester bonds based on difference in solubility.

Applicant wishes to note that the passages in Goodman et al. cited by the Examiner (i.e., column 3, lines 44-51 and 57-68, and column 4, lines 1-15) do not suggest how to facilitate ester bond formation over amide bond formation. The abovementioned passages merely discuss suitable reactant ratios, diluents and catalysts, without even remotely suggesting how these may affect ester bond formation versus amide bond formation.

Thus, the Examiner's statement appears to be that the technical difficulty of having to prevent amide bond formation in order to facilitate ester bond formation may be avoided by introducing the technical difficulty of separating polymers with sufficient ester bonds from a mixture of reaction products. Even if Goodman et al.

teaches how this may be achieved, this represents the introduction of a technical difficulty, as well as a reduction in yield, for no apparent reason.

Moreover, the solubility-based method taught by Goodman et al. is not taught therein as being effective for separating products obtained with a hydroxy amino acid. As would be apparent to one of ordinary skill in the art, hydroxy amino acid residues may have considerably different solubility characteristics than the hydroxyacid residues taught in Goodman et al., as the amino side chains of hydroxy amino acids are considerably different in their chemical properties than the alkyl and aryl side chains of the hydroxyacids taught by Goodman et al.

Applicant therefore believes that the modification of the hydroxyacids taught by Goodman et al. so as to include an amino group would render the hydroxyacids unsatisfactory for the purpose intended by Goodman et al.

As discussed in MPEP §2143.01, if the proposed modification would render the prior art invention being modified unsatisfactory for its intended purpose, then there is no suggestion or motivation to make the proposed modification. *In re Gordon*, 733 F.2d 900, 221 USPQ 1125 (Fed. Cir. 1984).

Applicant further argued in the above-mentioned response, filed June 23, 2008, that Goodman et al. teaches that the object of the linear polymer taught therein is to provide a polymeric material which is more hydrolyzable than polypeptides such as silk and collagen, and therefore more suitable for use in absorbable sutures, and that as is well known in the art, the introduction of branching and/or cross-linking of polymer molecules would be expected to make the polymer less hydrolyzable.

The Examiner has stated that this argument is unpersuasive because it is reasonable to believe that when the polymer is still soluble, the hydrolysability of the polymer will not be significantly changed by the presence of crosslinking until Applicant can present data to the contrary.

Applicant strongly believes that even if the hydrolysability of a **soluble** (Examiner's emphasis) polymer is not significantly affected by crosslinking, as suggested by the Examiner, this would not motivate one of ordinary skill in the art to modify the teachings of Goodman et al., because Goodman et al. teaches polymers

which are moderately hydrolysable under physiological conditions, which are characterized by high tensile strength, and which are useful for applications which require a solid substance, e.g. sutures, films, fibers (see, for example, column 1, lines 18-24 and 33-59, therein). Hence, one of ordinary skill in the art would not be motivated by the teachings of Goodman et al. to make a polymer which is both hydrolysable and soluble under physiological conditions.

Applicant respectfully submits herewith evidence to the above, which supports the above-indicated notion that one of ordinary skill in the art would expect the hydrolysability of the polymer to be decreased by the presence of crosslinking.

Thus, the Examiner's attention is respectfully directed to Vaz et al. (*Polymer Degradation and Stability* 2003, 81:65-74), a copy of which is enclosed herewith, who teach that resistance of soy-derived protein to non-enzymatic and enzymatic degradation is proportionate to the degree of crosslinking (see, for example, Abstract, as well as page 73, Conclusions, therein).

The Examiner's attention is further respectfully directed to U.S. Patent No. 4,947,840, a copy of which is enclosed herewith, which describes crosslink density as being generally inversely proportional to biodegradation rate (see, for example, column 4, lines 29-36, as well as column 3, lines 11-13, therein).

The Examiner's attention is further respectfully directed to Lutolf et al. (*Nature Biotechnology* 2003, 21:513-518), a copy of which is enclosed herewith, who teach that a PEG-peptide hydrogel matrix is degraded *in vivo* more rapidly when the degree of crosslinking is lower (see, for example, paragraph bridging pages 515 and 516 therein).

One of ordinary skill in the art would therefore expect the proposed modification of the teachings of Goodman et al. to reduce the biodegradability of the polymer.

Applicant therefore believes that it would be contrary to the stated object of the invention of Goodman et al. to modify the hydroxyacid residues taught by Goodman et al. so as to include a reactive functional group (e.g., an amino group) capable of inducing cross-linking of the polymer taught therein.

Applicant further argued in the above-mentioned response, filed June 23, 2008, that Goodman et al. teach away from the use of hydroxy amino acids by specifically defining the side groups of the polymer taught therein (i.e., R, R_1, R_2 and R_3) as being alkyl, aryl, aralkyl or alkaryl (see, for example, column 1, line 60, to column 2, line 12, therein). As is well known in the art, such functional groups are relatively non-reactive.

Applicant wishes to note that the Examiner has not responded to this argument in the Office Action.

The definition of the side groups of the polymer taught by Goodman et al. excludes numerous common natural amino acids (e.g., serine, lysine, glutamate, etc.) which have more reactive functional groups (e.g., carboxy, amine, hydroxy) in their side chains. Moreover, Goodman specifically describes numerous uncommon amino acids with non-reactive side chains as being suitable (see, for example, column 2, lines 55-64, therein).

As discussed hereinabove, the non-reactive side chains described by Goodman et al. avoid crosslinking, consistent with the purpose of the polymer as intended by Goodman et al.

As further discussed hereinabove, the absence of amine functional groups on the hydroxyacids described by Goodman et al. results, without creating technical difficulties, in a polymer with ester bonds, consistent with the purpose of the polymer as intended by Goodman et al.

In view of the above, it is argued that a person of ordinary skill in the art would therefore conclude that Goodman et al. deliberately defines the side chains of the polymer as being non-reactive groups, and specifically avoids using common amino acids with more reactive side chains, because Goodman et al. considers reactive side chains as being undesirable.

Goodman et al. therefore teaches away from the use of the hydroxy amino acids taught by Gouesnard, instead of hydroxyacids, as hydroxy amino acids would introduce reactive side chains (amines) to the polymer.

As further noted in Applicant's arguments in the above-mentioned response filed June 23, 2008, Goodman et al. teach a synthetic polymer preferably synthesized using synthetic derivatives of specific amino acids and hydroxyacids, while teaching away from using many common natural amino acids. One of ordinary skill in the art would therefore have no motivation to replace the hydroxyacids taught therein with modified natural amino acids.

In sharp contrast, the modification of natural amino acids is an advantageous feature of embodiments of the present invention, as according to these embodiments, natural protein sources can be utilized to produce a wide variety of polymers.

In view of the above, Applicant strongly believes that one of ordinary skill in the art would not have been motivated to modify the polymer taught by Goodman et al. by replacing the hydroxyacids taught therein with the hydroxy amino acids taught by Gouesnard.

Applicant therefore believes that claims 132-139 and 141-143 are not rendered obvious by Goodman et al. in view of Gouesnard, and are therefore allowable.

In view of the above remarks it is respectfully submitted that claims 132-139 and 141-143 are now in condition for allowance. A prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

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Date: March 16, 2009

Enclosures:

- Petition for Extension (Two Months)
- References:
  Vaz CM et al., *Polymer Degradation and Stability* 2003, 81:65-74
  Lutolf MP et al., *Nature Biotechnology* 2003, 21:513-518
  U.S. Patent No. 4,947,840

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In vitro degradation behaviour of biodegradable soy plastics: effects of crosslinking with glyoxal and thermal treatment

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Abstract

In-vitro degradation of soy-derived protein materials, non-crosslinked (SI_{tp}), crosslinked with glyoxal ($X\text{-}SI_{tp}$) or submitted to heat treatment ($24TT\text{-}SI_{tp}$), was studied with either an isotonic saline solution without enzymatic activity or containing bacterial collagenase. The changes in weight of the samples during the in-vitro degradation were studied and compared with the variations of the mechanical properties. The weight loss of SI_{tp}, $X\text{-}SI_{tp}$ and $24TT\text{-}SI_{tp}$ were more pronounced when using collagenase. After 24 h of immersion, SI_{tp} lost 10.6% of its initial weight whereas $0.6X\text{-}SI_{tp}$ and $24TT\text{-}SI_{tp}$ lost 1.7 and 5.7%, respectively. In every case, the weight loss was found to be directly proportional to the respective crosslinking degree: 2.4% for SI_{tp}, 44% for $0.6X\text{-}SI_{tp}$ and 27.8% for $24TT\text{-}SI_{tp}$. Consequently, the susceptibility of the soy materials towards enzymatic degradation could be controlled by varying the degree of crosslinking of the samples. The mechanical properties proved to be more sensitive to the loss of plasticiser (glycerol) during immersion than to the degradation of the polymeric matrices. After 24 h of immersion all the materials presented an increase in stiffness and brittleness due to the complete leaching of glycerol from the matrices. SI_{tp}, $X\text{-}SI_{tp}$ and $24TT\text{-}SI_{tp}$ proved to be suitable materials for either load-bearing applications or temporary applications such as tissue engineering scaffolds or drug delivery systems.

Keywords: Soy; Soy-derived protein material; Crosslinking; Mechanical properties; In-vitro degradation

1. Introduction

Several degradable polymers have been successfully used in biomedical applications. However, continuing research in the search for alternatives is suggested by some weaknesses of these materials, namely: i) the low level of degradability of some of these polymers, due to restrictions in their chemical nature [1–3]; and ii) eventual problems associated with the respective degradation products [4]. Furthermore, the demands for biomaterials with controlled and predictable degradation kinetics are numerous and have led to research on a variety of synthetic and natural polymers engineered for use in a wide range of medical applications.

During the last decades, aliphatic polyesters have been one of the most widely used classes of degradable polymers in the medical field [5–7]. Despite their wide applicability, many researchers are exploring new directions to address some of their current limitations, namely those related to their degradation mechanisms [4]. A variety of natural polymers, such as hyaluronic acids, alginates, starch and animal-origin proteins (namely, collagen, gelatine and albumin), are being extensively explored as potential biomaterials, particularly to be used in tissue regeneration scaffolds [8–10] and controlled release systems [11–15]. Among these naturally derived polymers, non-animal origin proteins, such as soy, may constitute a viable source of degradable materials for biomedical uses. In fact, they are expected to combine the main characteristics of collagen, gelatine and albumin with: i) a reduced susceptibility to thermal degradation (allowing for its easy processing by melt based technologies into complex 3D

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implants); and ii) an adequate degradation for the envisaged applications. Like the above described proteins, soy also proved to be non-cytotoxic, slightly immunogenic and bioactive when reinforced with bone like ceramics [16–18]. It has also been reported [19–22] that soy presents good processability, both in aqueous media and in the melt, and a reduced susceptibility to thermal degradation [23]. Soy is also expected to present an adequate degradation profile. In this case, it may be regarded as an eventual ideal template suitable for being used in biomedical applications. Temporary replacement implants, tissue engineering scaffolding or drug delivery systems are the most promising target applications.

However, due to the high enzymatic turnover rate of proteins in the human body, the stabilisation of protein-based materials, by crosslinking methods, is required to assure the respective integrity and the desired mechanical properties during an implantation period [24,25]. Crosslinks in proteins can be created by a number of ways, namely by physical or chemical routes [26]. The most used agents for chemical crosslinking are aldehydes, namely formaldehyde and glutaraldehyde. However, concerns related with the use of these two agents arose from an exacerbating effect on the calcification of prosthesis materials [27], cytotoxicity due to post-implantation depolymerization and monomer release from the crosslinked materials [28,29]. A promising solution for biomedical purposes is glyoxal, a dialdehyde with lower toxicity [30], when compared with similar agents. Other alternative which requires no crosslinking agents is based on the use of a physical method: thermal treatments.

This paper reports on the in vitro degradation behaviour of soy plastics using an isotonic saline solution with or without bacterial collagenase (degrading enzyme present in the human body and active during an implantation process). Soy plastics were previously crosslinked using glyoxal and a thermal treatment. The susceptibility of the resulting materials towards degradation is analysed in terms of the following aspects of the tested samples: i) weight; ii) crosslinking density; and iii) mechanical properties.

2. Materials and methods

2.1. Materials

Loders Crocklaan BV (Wormerveer, The Netherlands) supplied the non-GMO soy protein isolate (SI, 83.4% protein, w/w on dry weight base). Glycerol, glyoxal (40% v/v) and *o*-phthaldialdehyde (OPA) were used as received from the manufacturer, Sigma-Aldrich Chemie BV (Zwijndrecht, The Netherlands). NaCl, NaOH and HCl were all of analytical grades.

2.2. Production of soy specimens

Native soy protein was converted into a thermoplastic material (SI_{tp}) in a co-rotating twin-screw extruder as described previously in [22]. During the extrusion procedure, the soy protein was also crosslinked with different amounts of glyoxal, namely 0, 0.3 and 0.6% w/w based on the protein content (SI_{tp}, 0.3X-SI_{tp} and 0.6X-SI_{tp}, respectively). The extruded thermoplastic materials (in the form of pellets) were moulded into ASTM tensile test bars (2×4 mm^2 of cross section), as described in [22]. A batch of the injection-moulded specimens (SI_{tp}) was submitted to a thermal treatment performed at 80 °C during 24 h (24TT-SI_{tp}).

2.3. Degradation tests

2.3.1. Non-enzymatic degradation

Non-crosslinked and crosslinked samples were submitted to in vitro degradation tests. Pre-weighed dry specimens were immersed for 0, 1, 3, 5, 7 and 14 days, at 37 °C, in an isotonic saline solution [ISS—NaCl, 9 g/l + 1% sodium azide (NaN_3)] buffered at pH = 7.40 ± 0.02 with a 0.2 M tris(hydroxymethyl) aminomethane/0.2 M hydrochloric acid (HCl) buffer (0.2 M Tris–HCl buffer).

2.3.2. Enzymatic degradation with collagenase

The degradation of the non-crosslinked and the crosslinked soy samples was also evaluated using bacterial collagenase from Clostridium histolyticum (EC 3.4.24.3, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) with an activity of 283 U/mg (one unit releases peptides from native collagen equivalent in ninhydrin color to 1 μmol of L-leucine in 5 h at pH 7.4 at 37 °C in the presence of calcium ions). In a typical experiment, samples of the different soy materials were immersed for 0, 3, 6, 24 and 72 h, at 37 °C, in an isotonic saline solution (as described in Section 2.3.1) with collagenase (100 U/ml). The degradation was stopped at the desired time interval by addition of 0.2 ml of 0.25 M EDTA (Merck, Darmstadt, Germany) solution. The mixtures were cooled in ice. The remained soy specimens were washed with the Tris–HCl buffer and deionized water before determination of the respective weight loss.

2.4. Degradation products

2.4.1. Protein content

After ageing, samples were ground using liquid N_2 and subsequently sieved with a mesh size of 1 mm. 50 mg of protein samples was dispersed in a mixture of deionized water and concentrated H_2SO_4. After adequate digestion of the protein samples, carried out at 420 °C during 50 min, the total protein content (N_{tot}) of

the resulting solution was determined by Kjeldahl analysis and calculated as:

$$N_{tot}(\%) = [[V(HCl) * 0.1 * 14 * 6.25]/W_d] * 100 \quad (1)$$

where, V(HCl) is the volume of HCl 0.1 M used during the Kjeldahl titration, 14 is the atomic mass of nitrogen (N) and 6.25 the Kjeldahl factor of soy. W_d is the dry weight of the protein powder sample tested.

2.4.2. Free amino groups measurement

The free amino group content was determined using the OPA method [31] for all the samples aged for 0 and 24 h. An OPA solution was made by mixing 25 ml of 0.1 M sodium borate (pH 9.2), 2.5 ml of 20% (w/w) SDS and 40 mg of OPA (dissolved in 1 ml methanol) and 100 μl of β-mercaptoethanol. The final volume was adjusted to 50 ml with deionized water. To determine the degree of alkylation, an aliquot (50 μl containing 2 g/l protein in sodium tetraborate buffer 0.0125 M + 2% SDS) was added directly to 1.0 ml of OPA reagent in a cuvette. The solution was mixed rapidly and incubated for 2 min at room temperature before the absorbency was read at 340 nm against water. A calibration curve was previously established by using L-leucine as a standard.

2.4.3. Carboxylic groups measurement

An adapted titration was used for measuring the amount of terminal carboxylic groups in the soy materials [32].

0.33 g of noncrosslinked and crosslinked soy materials was dispersed in 50 ml of deionized water. An automatic titrator (Titronic 97/20) was used to adjust, under magnetic stirring, the pH of the solutions to 7 using 0.1 M HCl or 0.1 M NaOH in order to have the zwitterionic form of the protein in solution: $^{+}$3HN-RH-COO^{-}. It is assumed that the amounts of HCl needed to go from pH 6 to pH 3 ($^{+}$3HN-RH-COOH) correspond to the amount of carboxyl groups.

2.4.4. Buffer diffusion coefficients

To determine the buffer diffusion coefficients of the specimens, swelling tests were performed. The specimens were immersed up to 24 h in both degrading solutions described in Sections 2.3.1. and 2.3.2.

The wet weight of the specimens was determined by first blotting the specimen with filter paper to remove adsorbed buffer on the surface, and then weighed immediately on an electronic balance [33]. The following Eq. (2) was used to determine the buffer diffusion coefficient, D [34]:

$$W_t/W_e = 4 * [D/(\pi * h^2)]^{0.5} * t^{0.5} \quad (2)$$

where, W_t and W_e denote, respectively, the weight increase due to the buffer absorption at time t and at equilibrium time. D is the diffusion coefficient and, considering that absorption occurs from both sides of the sample, h is the overall thickness of the sample.

2.4.5. Weight loss

After each ageing period, the samples were removed from the degradation solution, washed with distilled water and dried in a vacuum oven (40 °C/24 h) [33]. The percentage weight loss of the soy materials was then calculated from Eq. (3):

$$WL_t = [(W_0 * N_0 - W_t * N_t)/(W_0 * N_0)] * 100 \quad (3)$$

where WL_t is the weight loss of after a certain time t of immersion. W_t denotes the weight of the specimen at ageing time t and N_t the respective protein content at that ageing time, W_0 is the initial dry weight of the specimen and N_0 the correspondent protein content. Each experiment was repeated three times and the average value was taken as the weight loss.

2.4.6. Dimensional variation

Before tensile testing, all the aged specimens were accurately measured for its thickness (h). This allowed for the evaluation of the surface erosion of the specimens as a function of immersion time.

2.4.7. Mechanical properties

Crosslinked and non-crosslinked samples aged for 0, 3, 6, 24 and 72 h in the isotonic saline solutions with and without enzymatic activity where conditioned for 1 week in a chamber at 25 °C and 90% relative humidity before testing for its mechanical properties. The measurements were assessed in tensile mode. The experiments were performed in a Zwick Z010 universal mechanical testing machine, in a controlled chamber (20 °C and 55% RH). A 5 kN load cell, a pre-load of 0.1N and a loading speed of 1 mm/min were used. E-modulus at 0.05–0.25% strain ($E_{0.05-0.25\%}$), yield stress (σ_y) and strain at break (ε_b) were computed from the respective data.

2.4.8. Moisture content

After tensile testing, specimens were milled using liquid N_2 and weighed into aluminium dishes for subsequent drying for 24 h in a vacuum oven at 40 °C [33]. Moisture content (MC) was determined in triplicate for each type of material as percentage of initial weight (W_0) lost during drying (W_{0d}):

$$MC = [(W_0 - W_{0d})/W_0] * 100 \quad (4)$$

2.4.9. Molecular weight distributions

To a SDS-PAGE sample (containing at least 50 mg of the specimen degraded for 0, 3 and 14 days), 6 ml of electrophoresis buffer (50 mM Tris–HCl pH 6.8, 12%

glycerol w/v, 4% SDS w/v, 2% β-mercaptoethanol w/v and bromophenyl blue) was added and left standing at room temperature for 2 h with vortexing every 15 min. Subsequently, the mixture was centrifuged at 8000×*g* for 10 min. The supernatant was kept at −20°C for later electrophoresis.

Before electrophoresis, the supernatant was boiled for 5 min to break the S–S bonds. Subsequently, it was cooled down and applied to a gel prepared from 15% acrylamide. High molecular standards (phosphorylase b: 94,000 D; BSA: 67,000 D; ovalbumin: 43,000 D; carbonic anhydrase: 30,000 D; soybean trypsin inhibitor: 20,100 D; α-lactalbumin: 14,437 D) from Pharmacia (Uppsala, Sweden) were used as protein references. The electrophoresis was conducted using a II Dual Slab Cell system (Bio-Rad, Veenendal, The Netherlands) at a voltage of 150 V which was increased to 200 V when the protein reached the dividing line between the stacking and the separating gels. The gels were stained using Serva Blue R for 45 min and destained by immersion in a solution of methanol:acetic acid:dionized water (4:1:5) for at least 3 h. After decolouration, the gels were dried in a coating dryer (Bio-Rad Laboratories) for 1 h at 60 °C.

3. Results

3.1. Weight loss

3.1.1. Glyoxal crosslinking

The crosslinking of the soy samples with glyoxal was related to the correspondent in-vitro degradation behaviour. First, the number of free carboxyl and free amine groups of the developed compounds were determined to quantify the crosslinking density (Table 1).

The results confirmed that glyoxal crosslinking occurred via the free amine groups of the lysine (or hydroxylysine) residues, which decrement is observed for higher amounts of the crosslinking agent

The weight loss of the materials with different degrees of crosslinking, as a function of degradation time, is presented in Figs. 1 and 2.

Table 1
The content of amine and carboxyl groups of soy crosslinked with glyoxal and heat treated soy

Materials	Free amine group content (%)	Carboxyl group content (mmol/100 g protein)
SI_{tp}	97.6±0.5	108.3±0.002
0.3X-SI_{tp}	66.3±1.1	110.5±0.009
0.6X-SI_{tp}	55.9±1.0	111.3±0.003
24TT-SI_{tp}	72.2±1.1	112.1±0.007

SI_{tp}: soy protein thermoplastic; #X-SI_{tp}: soy protein thermoplastic crosslinked with #% glyoxal (% based on the protein content); 24TT-SI_{tp}: heat treated soy protein thermoplastics.

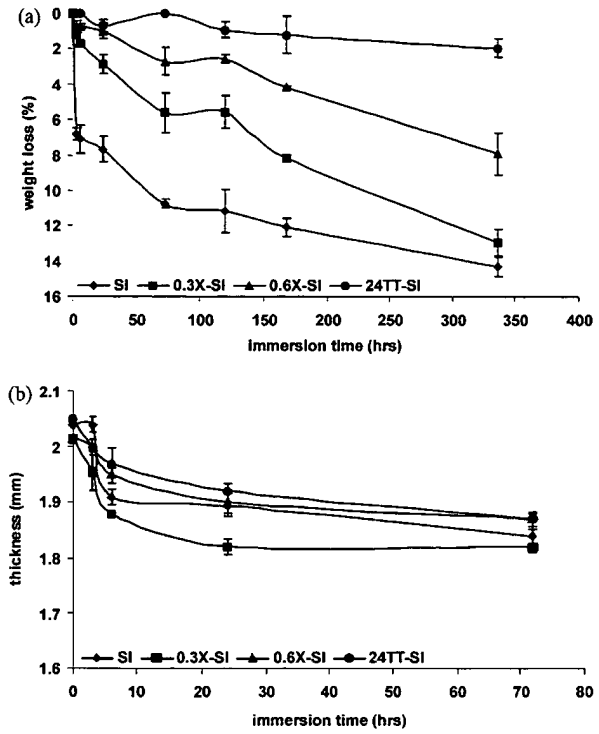

Fig. 1. Changes in (a) weight and (b) thickness of soy protein based materials as a function of degradation time during exposure to an isotonic saline solution (NaCl 9 g/l, pH 7.4 and 37 °C).

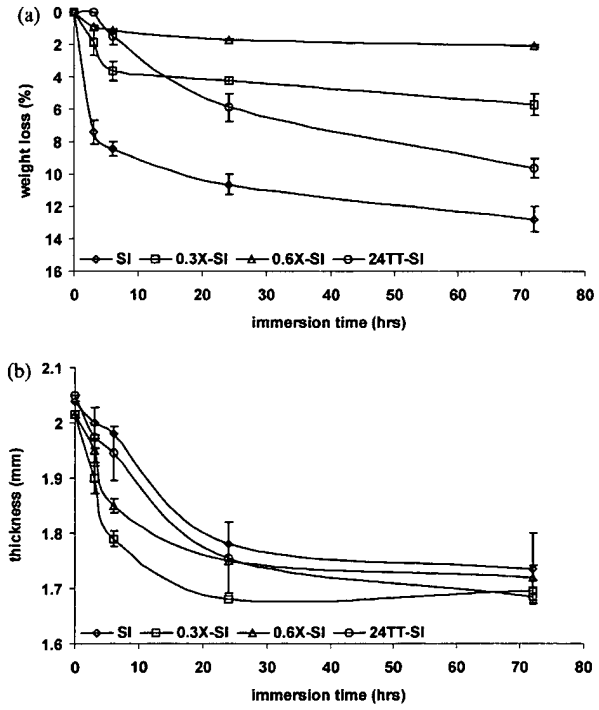

Fig. 2. Changes in (a) weight and (b) thickness of soy protein based materials as a function of degradation time during exposure to an isotonic saline solution with bacterial collagenase (100 U/ml, pH 7.4 and 37 °C).

Two different immersion media were used: i) without enzymes; and ii) with bacterial collagenase, respectively. The material having the highest degree of crosslinking showed the highest resistance against hydrolysis and the lowest coefficients of diffusion (Table 2).

An almost linear decrease in the material weight loss was observed for different degrees of crosslinking (Fig. 3).

Although the diffusion coefficients of the enzymatic solution were always lower than those of the non-enzymatic one (Table 2), all the materials showed a slightly higher susceptibility towards degradation by collagenase. The weight loss rates, determined from the slopes of the initial linear part of the curves displayed in Fig. 1, are presented in Fig. 4 as a function of the amount of free amine groups in the protein.

A decrease in free amine groups results in a lower degradation rate. It appears that a crosslinking degree of 50% must be reached, above which only slow degradation is observed in both media.

Table 2
Diffusion coefficients of the tested degradation solutions into the (non)-crosslinked and heat treated soy samples

Materials	$D\times10^{-8}$ (cm^2/s)	
	ISS	ISS + collagenase
SI_{tp}	62.5	59.1
0.6X-SI_{tp}	46.0	42.1
24TT-SI_{tp}	52.6	48.4

SI_{tp}: soy protein thermoplastic; #X-SI_{tp}: soy protein thermoplastic crosslinked with #% glyoxal (% based on the protein content); 24TT-SI_{tp}: heat treated soy protein thermoplastics; *D*: diffusion coefficient; ISS: isotonic saline solution, NaCl 9g/l, buffered at pH 7.4 with 0.2 M Tris–HCl.

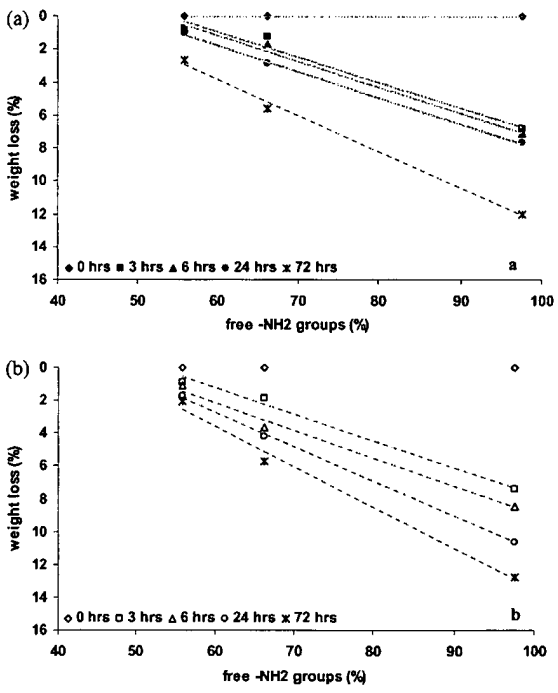

Fig. 3. Change in weight of soy protein based materials as a function of the respective crosslinking degree in (a) isotonic saline solution (9 g/l NaCl, pH 7.4, 37 °C) or (b) isotonic saline solution with bacterial collagenase (100 U/ml, pH 7.4, 37 °C).

3.1.2. Heat treatment

The free amine group and the carboxyl contents of the heat treated soy materials are given in Table 1. Fig. 5

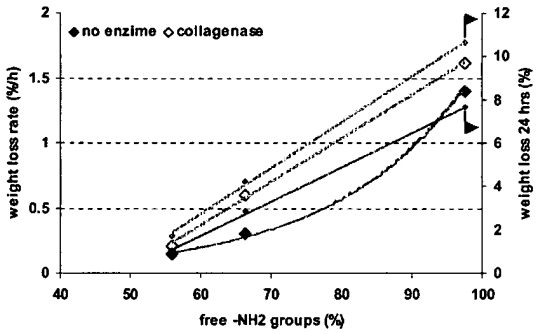

Fig. 4. Rate of weight loss (◆,◇) and weight loss for 24 h (◆,◇) of soy crosslinked with 0, 0.3 and 0.6% glyoxal (SI_{tp}, 0.3X-SI_{tp} and 0.6X-SI_{tp}, respectively) during immersion in an isotonic saline solution (◆, ◆, 9 g/l NaCl, pH 7.4, 37 °C) or in an isotonic saline solution with bacterial collagenase (◇,◇, 100 U/ml, pH 7.4, 37 °C) as a function of the degree of crosslinking of the tested materials.

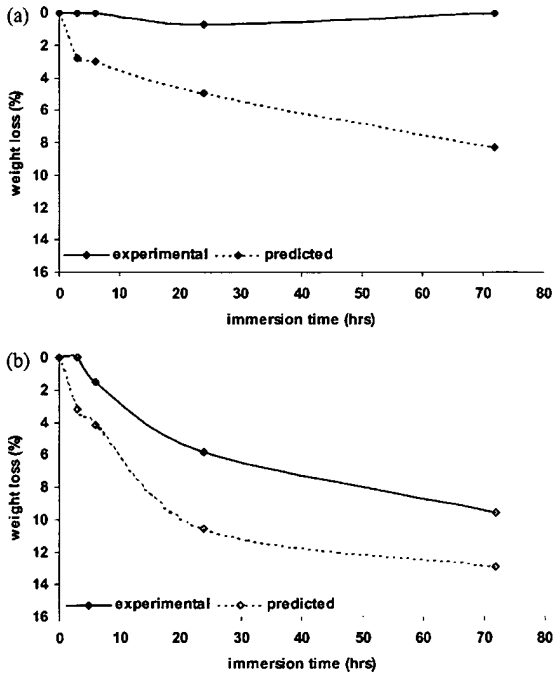

Fig. 5. Experimental and theoretically expected change in weight of the heat treated soy (24TT-SI_{tp}) tested in (a) isotonic saline solution (9 g/l NaCl, pH 7.4, 37 °C) or (b) isotonic saline solution with bacterial collagenase (100 U/ml, pH 7.4, 37 °C), considering the respective crosslinking degree.

presents the weight loss of the heat treated soy plotted as a function of the immersion time. The represented lines were determined in two different ways: i) experimental: using the procedure described in Section 2.3.3; and ii) predicted: only considering the effect of the amount of free amine groups (using the linear curve fitting of the results presented in Fig. 3).

The heat treated materials present a smaller susceptibility towards non-enzymatic degradation than the glyoxal crosslinked ones (Fig. 1a). By the contrary, when submitted to enzymatic media showed faster release rates (Fig. 2a). From the results presented in Fig. 5 it is possible to conclude that the crosslinking through the amine groups of soy should not be the only mechanism responsible for the degradation behaviour of the heat treated soy materials. The experimental degradation rates observed for heat treated soy are always slower than the theoretical predictions based only on the amine groups reactions (Fig. 5).

3.2. Mechanical properties

The change in mechanical properties of non-crosslinked soy (SI_{tp}), soy crosslinked with glyoxal (0.6X-SI_{tp}) and heat treated soy (24TT-SI_{tp}) as function of degradation time was evaluated. The content of amine groups and the mechanical properties of the soy materials are presented in Table 3.

The mechanical properties of the soy materials depend on the crosslinking methodology. Crosslinking with glyoxal (0.6X-SI_{tp}) resulted in materials which have a decreased tensile strength and strain at break and an increased stiffness as compared to SI_{tp}. Heat treatment afforded a material with a higher tensile strength and stiffness and lower strain at break as compared to SI_{tp}.

3.2.1. Non-enzymatic degradation

After 24 h of immersion, non-crosslinked soy (SI_{tp}) retained over 92% of its original weight, whereas a 1% and 0.7% decrease in weight were observed for glyoxal crosslinked soy (0.6X-SI_{tp}) and heat treated soy (24TT-SI_{tp}), respectively. During degradation, the free amine group content of SI_{tp} increased from 97.6 to 100%, from 55.9 to 60.5% for 0.6X-SI_{tp} and from 72.2 to 80.2% for 24TT-SI_{tp}.

The mechanical properties of SI_{tp}, 0.6X-SI_{tp} and 24TT-SI_{tp} were reasonably affected upon degradation, as shown in Fig. 6.

An increase of the E-modulus was observed for all materials. The tensile strength also increased from 3.5 to 8.7 MPa for SI_{tp} and from 3.9 to 9.4 MPa for 24TT-SI_{tp}. By the contrary, glyoxal crosslinked soy showed a slight decrease in this property (from 3.1 to 2 MPa). Furthermore, the strain at break was highly decreased from: i) 36.4–5.5% for SI_{tp}; ii) 25.5–1.8% for 0.6X-SI_{tp}; and iii) 21.7–9.8% for 24TT-SI_{tp}.

3.2.2. Collagenase degradation

The effect of collagenase on the mechanical properties of SI_{tp}, 0.6X-SI_{tp} and 24TT-SI_{tp}, as a function of immersion time, is presented in Fig. 7.

After 24 h of immersion, non-crosslinked soy (SI_{tp}) retained over 89% of its original weight, whereas a 1.7% decrease in weight was obtained for glyoxal crosslinked soy (0.6X-SI_{tp}). The 24TT-SI_{tp} material seemed to be the most affected by the collagenase action. After 24 h of immersion, it suffered a decrease of 6% in weight compared to the only 0.7% decrease observed during the non-enzymatic tests. During degradation, the free amine group content of SI_{tp} increased from 97.6 to 100%, from 55.9 to 68.1% for 0.6X-SI_{tp} and from 72.2 to 97.7% for 24TT-SI_{tp}.

The mechanical properties of SI_{tp} and 0.6X-SI_{tp} were not further affected by the presence of collagenase. By the contrary, heat treated soy was the most affected by the enzymatic action. It was observed a lower increase in stiffness and strength when compared with those observed during immersion in the non-enzymatic medium. The strain at break also decreased. It was observed a decrease from 21.7 to 9.8% for the non-enzymatic medium compared with a decrease from 21.7 to 5.3% for the medium with collagenase.

3.3. Molecular weight distributions

SDS-PAGE patterns of the soy thermoplastics (SI_{tp}, 0.6X-SI_{tp} and 24TT-SI_{tp}) were obtained to examine the

Table 3
Amine group content and mechanical properties of (non)-crosslinked and heat treated soy

Material	Free–NH_2 group content (%)	Tensile strength (MPa)	Strain at break (%)	$E_{0.05-0.25\%}$-modulus (MPa)	Moisture content (%)
SI_{tp}	97.6±0.5	3.5±0.3	36.4±0.5	33±10	16.8±0.2
0.6X-SI_{tp}	55.9±1.0	3.1±0.1	25.5±4.7	68±1	16.3±0.1
24TT-SI_{tp}	72.2±1.1	3.9±0.1	21.7±2.1	88±3	14.6±0.4

SI_{tp}: soy protein thermoplastic; 0.6X-SI_{tp}: soy protein thermoplastic crosslinked with 0.6% glyoxal (% based on the protein content); 24TT-SI_{tp}: heat treated soy protein thermoplastics.

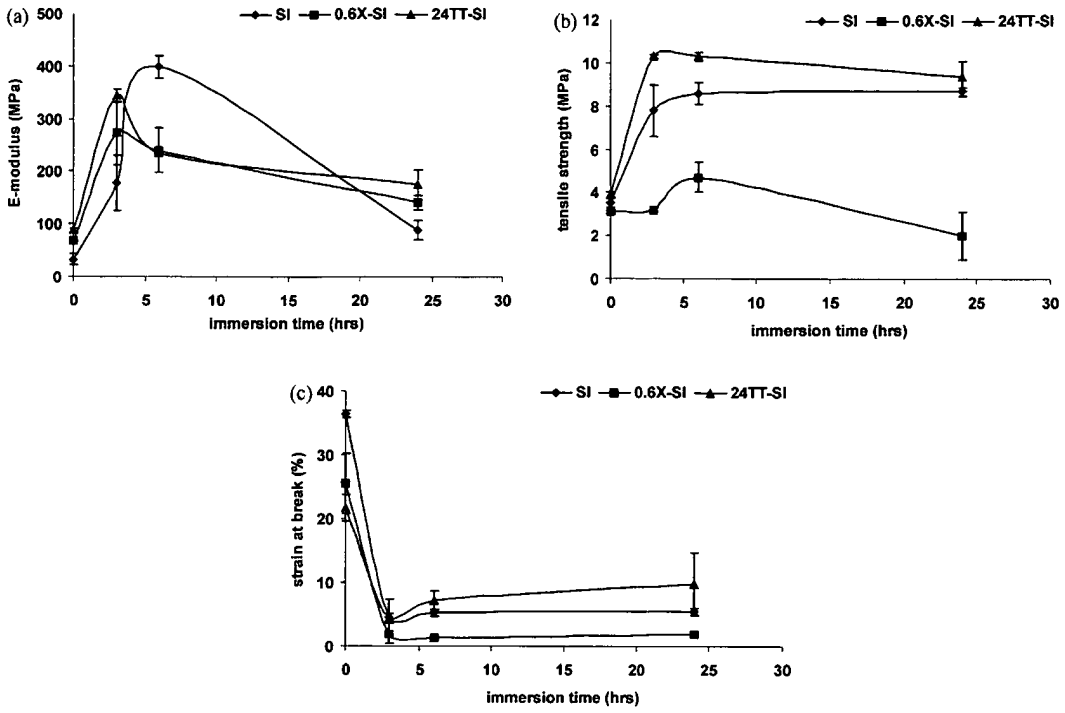

Fig. 6. Changes in (a) E-modulus, (b) tensile strength and (c) strain at break as a function of degradation time during exposure to an isotonic saline solution (NaCl 9g/l, pH 7.4 and 37 °C).

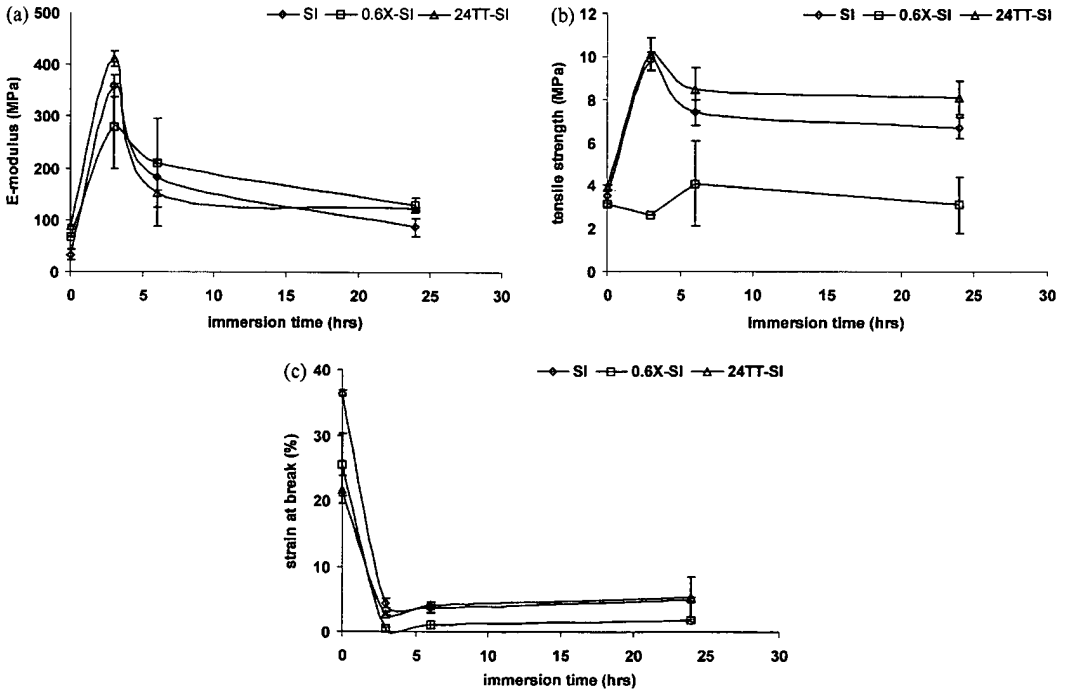

Fig. 7. Changes in (a) E-modulus, (b) tensile strength and (c) strain at break as a function of degradation time during exposure to an isotonic saline solution with bacterial collagenase (100 U/ml, pH 7.4 and 37 °C).

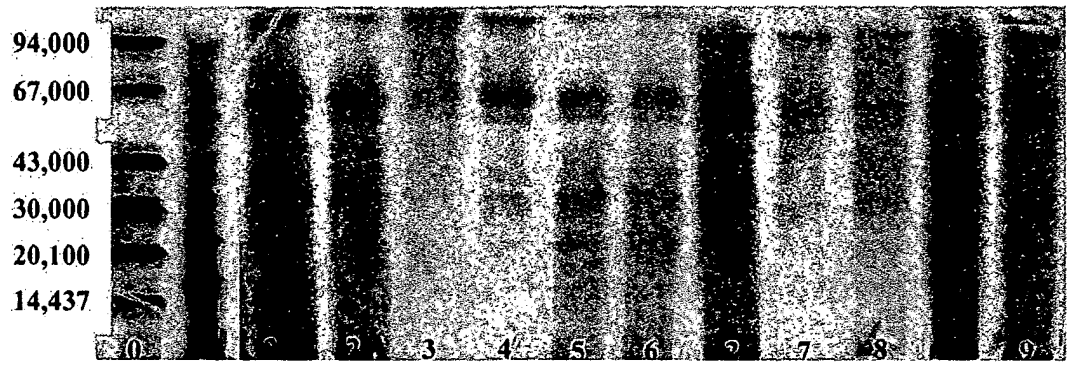

Fig. 8. SDS-PAGE results for standard: (0) protein markers; (1) native soy isolate; (2) thermoplastic soy (SI_{tp}); (3) heat treated soy (24TT-SI_{tp}); (4) glyoxal crosslinked soy (0.6X-SI_{tp}); (5) SI_{tp} after 3 days immersion; (6) 24TT-SI_{tp} after 3 days immersion; (7) 0.6X-SI_{tp} after 3 days immersion; (8) SI_{tp} after 14 days immersion; (9) 24TT-SI_{tp} after 14 days immersion in an isotonic saline solution (NaCl 9 g/l, pH 7.4 and 37 °C).

molecular weight distributions of the proteins after different degradation periods (Fig. 8).

All the thermoplastics soy materials presented a large band on the top of the gel ($M_w > 94{,}000$ D) which was absent in the soy isolate. Thus, the average molecular weight was slightly increased by extrusion and injection moulding operations, indicating that some intermolecular bonds were formed during processing. Glyoxal crosslinked samples (0.6X-SI_{tp}) showed a very weak intensity, partly reflecting the low solubility of this protein in the electrophoresis. Heat treated samples (24TT-SI_{tp}) also showed a band intensity weaker than SI but stronger than 0.6X-SI_{tp}, proving its lower degree of crosslinking. After degradation, all the protein patterns showed a decrease of intensity of the fractions correspondent to a lower Mw, and reflecting the preferential leaching of these fractions during the degradation tests. This observation was specially visible for SI_{tp} confirming its higher susceptibility to degradation than the correspondent crosslinked matrices.

4. Discussion

The degradation behaviour of protein-based materials can be controlled by the respective degree of crosslinking (induced by chemical or physical methods). Traditionally, glutaraldehyde, carbodiimides and epoxy compounds have been extensively used as crosslinking agents to stabilise arteries and pericardial heart valves [35–37].

The resistance against degradation of protein based materials can be studied by in-vitro tests, in isotonic saline solutions, including (or not) enzymes such as bacterial collagenase, and is mainly monitored by changes in weight as a function of the immersion time. The selected collagenase enzyme from Clostridium histolyticum is capable of cleaving peptide bonds within the protein structure and has a specificity for the Pro-X-Gly-Pro-Y region, splitting between X and Gly where X and Y are predominantly apolar amino acid residues. So, the degradation rate of the protein-based material will be mainly determined by the crosslinking density, the accessibility of the cleavage sites and the extent of denaturation [24,25]. Changes in the mechanical properties of the materials also become important when they are intended to withstand loads during implantation. Therefore, the influence of degradation on the mechanical performance of the non-crosslinked and crosslinked soy was also evaluated.

Treatment of soy with glyoxal or exposure to heat decreased the free amine group content of the material, evidencing the role of these groups on the crosslinking reactions (Table 1). The lowest free amine group content was observed for soy crosslinked with 0.6% glyoxal (0.6X-SI_{tp}). Furthermore, the free amine group contents vary from 97.6% for SI_{tp}, to 55.9% and 72.2% for 0.6X-SI_{tp} and 24TT-SI_{tp}, respectively. However, the behaviour of each material (Figs. 1 and 2) should be related not only with its degree of crosslinking but also with the respective nature.

The use of glyoxal leads to the crosslinking between the free amine groups of soy and the aldehyde groups of glyoxal. As previously discussed [22], the resulting crosslinks are mainly of intramolecular nature. Thus, the degradation behaviour of the glyoxal treated materials should be mainly related with their intramolecular crosslinking density. As observed in Fig. 3, the weight loss of these crosslinked samples was always directly proportional to its crosslinking density and always substantially lower than SI_{tp} specimens (Figs. 1 and 2).

The heat treatment of the soy materials results in the establishment of: i) crosslinks with the free amine groups (intramolecular crosslinking); ii) disulphide bonding (of intermolecular nature [22]); iii) hydrogen bonding; and iv) hydrophobic interactions. Consequently, the degradation behaviour of the heat treated materials (Figs. 1 and 2) should result from the combined effect of the respective intra- and intermolecular crosslinking density and degree of hydrophobicity. For

this reason, in both media (Fig. 5), these materials always present degradation rates slower than those predicted only taking into considering the contribution of the intramolecular crosslinks.

It has been shown that enzymatic degradation of polyesters occurs by a surface erosion process which is experimentally characterized by a zero order weight loss in the initial stage of the degradation [38]. A zero order weight loss was always observed in this work for the degradation of either non-crosslinked or crosslinked soy, during the first hours of immersion (Figs. 1a and 2a). This suggests that the degradation of soy materials can be depicted as a surface erosion process. These conclusions can also be supported by the observed decrease in the thickness of the tested bars as a function of immersion time (Figs. 1b and 2b), specially during the enzymatic degradation (Fig. 2b). In general, the decrease in the overall samples thickness corresponded to the percentage of material lost due to degradation (Figs. 1 and 2).

The very important changes in mechanical properties during the immersion period (Figs. 6 and 7) revealed a clear two stage process. Furthermore, it is also evident that this behaviour is the result of other contribution than only the degradation (and consequent weight loss) of the soy matrix.

In fact, if crosslinking prevents the penetration of the degrading solution in the protein matrix and only surface erosion occurs, the crosslinked materials would be able to retain the strength for a longer period during degradation. However, it was observed an increase in both strength and stiffness of the materials, accompanied by a sharp decrease in strain at break. As reported previously for other biopolymers containing glycerol [39], the degradation of these polymeric systems always involves the leaching of the plasticiser. So, the evidenced mechanical behaviour can be divided in two distinct phases:

a) First, glycerol is leached during the first three hours of immersion and masked the effects of the matrix degradation on the mechanical properties of the materials. This process is followed by an increase in the material stiffness and a severe loss of ductility (the strain at break drops to values between 1 and 6%);
b) The behaviour in the second stage, after 3 h of immersion, should be explained by the following mechanisms: i) as the matrix degradation occurs it enables a moisture uptake by the materials, which can slightly compensate the glycerol loss; ii) so, the strength and stiffness decrease and tend to stabilise, what is followed by a small increment and stabilization of the ductility (Figs. 6c and 7c); iii) these effects are less pronounced in the crosslinked materials because the water uptake of the respective macromolecular structures is more restricted; in fact, glyoxal crosslinked materials (0.6X-SI_{tp}) are more stable than the thermal treated ones (24TT-SI_{tp}) because the intramolecular crosslinks are important on preventing the referred water uptaking process; and iv) comparison between Figs. 6 and 7 show that, in general terms, the above referred processes are enhanced when the degradation occurs in the presence of enzymatic action.

5. Conclusions

Crosslinking of SI_{tp} with glyoxal (X-SI_{tp}) or by heat treatment (24TT-SI_{tp}) result in materials with different characteristics concerning mechanical properties and in-vitro stability. The susceptibility of the materials towards (non-) or enzymatic degradation could be controlled by varying the degree of crosslinking or hydrophobicity of the samples. Different degrees of crosslinking could be achieved using glyoxal or heat treatment. Samples crosslinked using the former method seemed to be more resistant toward degradation by a solution of bacterial collagenase. Contrarily, heat treated samples proved to be more resistant to degradation in the absence of collagenase. Upon degradation, the change in mechanical properties of SI_{tp}, 0.6X-SI_{tp} and 24TT-SI_{tp} samples were more sensitive to the leaching of glycerol than to matrices degradation. In general, soy thermoplastics are slightly sensitive to degradation with collagenase.

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Repair of bone defects using synthetic mimetics of collagenous extracellular matrices

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We have engineered synthetic poly(ethylene glycol) (PEG)–based hydrogels as cell-ingrowth matrices for *in situ* bone regeneration. These networks contain a combination of pendant oligopeptide ligands for cell adhesion (RGDSP) and substrates for matrix metalloproteinase (MMP) as linkers between PEG chains. Primary human fibroblasts were shown to migrate within these matrices by integrin- and MMP-dependent mechanisms. Gels used to deliver recombinant human bone morphogenetic protein-2 (rhBMP-2) to the site of critical-sized defects in rat crania were completely infiltrated by cells and were remodeled into bony tissue within five weeks. Bone regeneration was dependent on the proteolytic sensitivity of the matrices and their architecture. The cell-mediated proteolytic invasiveness of the gels and entrapment of rhBMP-2 resulted in efficient and highly localized bone regeneration.

Although autologous bone grafts are routinely used to heal large bone defects, the disadvantages of this intervention (e.g., limited graft quantity, donor site morbidity) continue to drive the development of improved methods for bone regeneration. A promising alternative to autografting is the delivery of osteoinductive growth factors, such as members of the BMP family. BMPs, powerful regulators of many cell functions both in the embryonic and adult organism[1], are capable of forming *de novo* bone by acting on osteoprogenitor cells and inducing them to differentiate into mature osteoblasts[2,3]. Recombinant human forms of these proteins have been produced[4], and some BMPs, in particular BMP-2 and BMP-7, have been shown to induce bone formation in various animal models and in a recent human clinical study[5].

The efficient clinical use of BMPs depends critically on the delivery strategy: when administered in solution, BMPs will be rapidly cleared, resulting in suboptimal healing. The use of biomaterials that can retain and sequester BMPs greatly enhances efficacy and reduces protein dose by localizing the morphogenetic stimulus[6–8].

Both naturally derived and synthetic materials have been extensively tested as BMP carriers for bone regeneration. Collagen can bind BMPs to some degree and is readily infiltrated and remodeled into bony tissue[9,10]. However, like other natural polymers, it has some limitations in clinical use, primarily because of handling problems, the difficulty of engineering its properties, and its immunogenicity[11,12]. Among the synthetic BMP carriers, hydrolytically degrading polymers made from lactide and glycolide monomers have been applied most frequently. These traditional polymers can be problematic because they acidify and can form proinflammatory fragments upon degradation[13–16]. New formulations of these and related bulk-degrading polymers[17–20] may overcome some of these problems.

With very few exceptions, the design of synthetic biomaterials used for bone regeneration has been based upon structural and biomechanical criteria and upon passive mechanisms of growth factor delivery. The complex biological molecular interactions between cells and the extracellular matrix (ECM) have been largely ignored. The goal of this work was to develop a class of synthetic materials—designed on the basis of biological recognition principles[21–25]—that could mimic some of the key characteristics of biologically derived components of the ECM, such as collagen. Besides providing structural and biochemical cues for cells in contact, an important characteristic of the ECM is its susceptibility to cell-triggered proteolysis, which enables cell invasion and subsequent remodeling of the matrix, leading to regeneration[26]. At least three members of the MMP family[27,28]—MMP-2, MMP-9, and MT1-MMP—have been reported to play a major role in bone development and remodeling[28,29].

To mimic MMP-mediated matrix turnover, we engineered synthetic hydrogels containing a combination of crosslinking MMP substrates and pendant adhesion sites. Using *in vitro* and *in vivo* assays, we demonstrate that these bioactive networks can undergo cell-mediated proteolytic degradation, leading to further remodeling into a cell-secreted bone matrix at the site of an injury. Analysis of bone healing in a critical-sized defect in the rat calvarium by microcomputed tomography showed that our biomimetic matrix performed as well as bovine collagen matrix with regard to the degree of closure, bone volume, and bone connectivity (at 5 μg BMP-2 per defect). Thus, it was possible to mimic the invasion-conducting capacity of collagen without the use of the xenogeneic bovine protein carrier and its associated immunological complexities.

Results and discussion

Newly developed biomaterials intended for clinical use should be easy to handle and suitable for *in situ* application. Toward this end, we developed a synthesis scheme based on conjugate addition reactions between conjugated unsaturations on end-functionalized PEG macromers and thiol-bearing peptides that allows formation of

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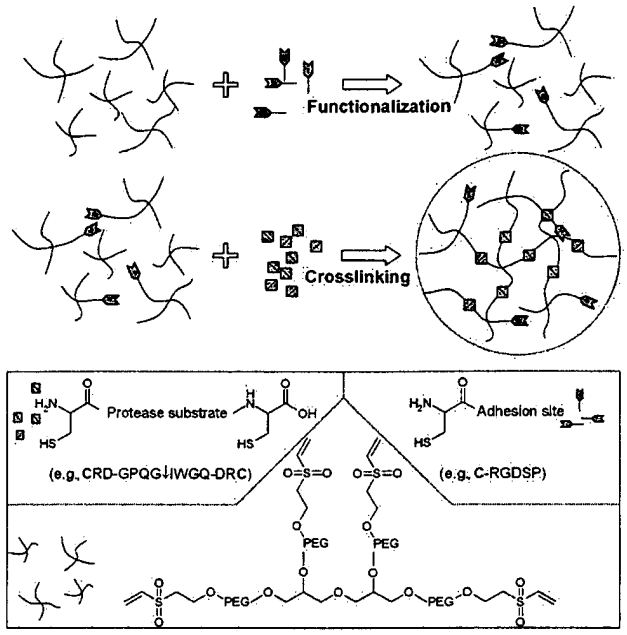

Figure 1. Scheme for gel preparation. Gel preparation procedure by selective conjugate addition. First a mono-functional peptide containing an integrin-binding RGDSP ligand for cell adhesion is reacted pendantly with a precursor containing multi-armed end-functionalized PEG macromers. Then the crosslinking is performed by the use of a bifunctional peptide. The bifunctional peptide determines the response of the material in the presence of cell-secreted enzymes, in this case, MMPs. As a result, these building blocks lead to the formation of elastic gel networks, to which cells can adhere, and which degrade to soluble products upon exposure to MMPs by cleavage of the crosslinking peptides.

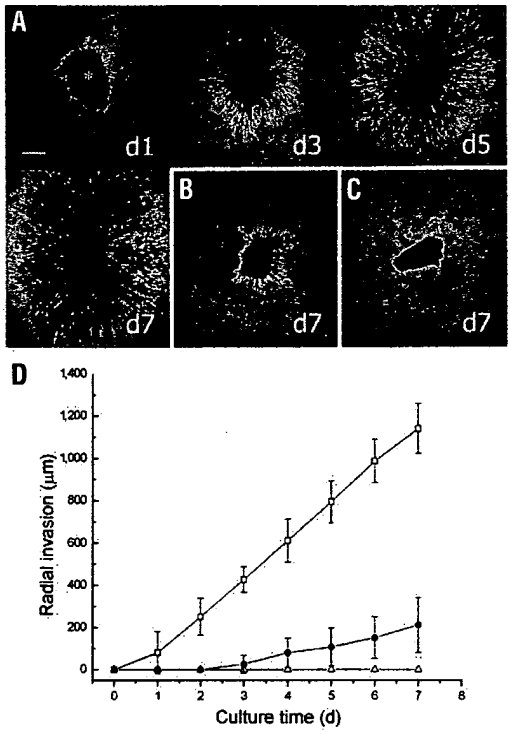

Figure 2. Three-dimensional fibroblast migration. (A) Fibroblasts migrate out in three dimensions from a cell-fibrin cluster (marked with *) into adhesive and MMP-sensitive synthetic matrices (corresponding to 1, 3, 5 and 7 d of culture of the same sample; □ in D). Cell invasion distances increase approximately linearly with culture time up to one week (n = 9–12). (B) In the presence of a broad-spectrum MMP inhibitor, hFF invasion was significantly reduced ($P < 0.01$) (day 7; ● in D), indicating that proteolysis was the main factor responsible for the outgrowth of cells. (C) Adhesion ligands were necessary for invasion to occur (day 7; △ in D). (D) Radial invasion of fibroblasts. Scale bar, 200 μm.

bioactive networks by the mixing of aqueous buffered solutions under almost physiologic conditions (Fig. 1).

In vitro fibroblast invasion. We investigated the sensitivity of the gels to proteolysis by cell-secreted MMPs using an *in vitro* model system for cell invasion. Clusters consisting of primary human foreskin fibroblasts (hFFs) entrapped in a fibrin matrix were polymerized within PEG gels, and three-dimensional (3-D) outgrowth was assessed microscopically. Spindle-shaped fibroblasts migrated radially out from clusters into the surrounding PEG matrix at an invasion rate of approximately 7 μm/hour (Fig. 2). Invasion was absent, as expected, within networks lacking the RGD ligand or containing an inactive RDG control motif (Fig. 2C), as a result of the lack of biological recognition of PEG[30]. Based on swelling measurements, the network mesh size (indicative of the distance between consecutive crosslinks) of these networks was approximately 30–50 nm, that is, well below the dimensions of cellular processes. Thus, 3-D migration was limited to mechanisms involving proteolytic degradation. Indeed, very little or no invasion was observed when a broad-spectrum MMP inhibitor was added to the culture medium (Fig. 2B,D). Outgrowth was similarly hindered in gels composed of a crosslinking peptide without an MMP substrate, providing additional evidence that the synthetic matrix was predominantly degraded by cell-associated MMPs.

Release of rhBMP-2 from MMP-sensitive PEG gels and collagen sponges. In developing gels as carriers for BMP-2, we noted that the kinetics of protein release can greatly influence bone regeneration. Therefore, we studied the release of rhBMP-2 from synthetic PEG-based matrices *in vitro* using previously described methods[31], and compared it with release from a widely used collagen sponge (Helistat). Collagen retained ~85% of the absorbed 5 μg rhBMP-2 after 12 hours and 60% after 60 hours of washing, with rhBMP-2 continuing to be released at that time (Fig. 3). By contrast, synthetic PEG-based gels retained ~90% of the protein after 60 hours of incubation and the release curve had already leveled off after ~24 hours. The solubility of rhBMP-2 in physiological saline at pH 7.4 is relatively low, ~60 μg/ml[32], and it may be that this relatively low solubility in combination with the PEG present in the gel network keep the protein in a precipitated state until gel dissolution[32–34]. As the size limit for diffusion in these networks substantially exceeds the Stokes' radius of rhBMP-2, it seems unlikely that protein diffusion is physically hindered. It is also unlikely that the rhBMP-2 becomes covalently coupled to the gel, because the crosslinking reaction has been demonstrated to be highly selective[35]. Importantly, the release of the entrapped rhBMP-2 from the PEG networks was achieved upon exposure to MMP-2 (Fig. 3), mimicking the proteolytic activity associated with invading cells. This suggests that local proteolytic activity released entrapped rhBMP-2 from the gel matrix.

Healing of critical-size rat calvarial defects. We tested the synthetic hydrogel matrices in critical-size calvarial defects of the rat (with 5 μg rhBMP-2 per implant volume of 100 μl). After five weeks, animals were killed, and harvested cranial samples were analyzed histologically (Fig. 4A–D) and by microcomputed tomography (Figs. 4E,F and 5). Synthetic hydrogels that were adhesive and MMP-sensitive and that contained rhBMP-2 showed intramembranous bone formation (calcified bone stained in green with Goldner Trichrome) and osteoblasts (fuschia) at the osteoid interface between the newly deposited calcified bone and the fibroblast-like cells initially infiltrating the matrix

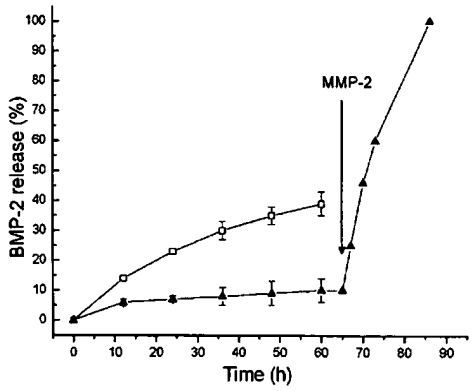

Figure 3. rhBMP-2 release triggered by MMP activity. Retention of physically entrapped and adsorbed rhBMP-2 (5 µg per 100 µl sample) in PEG gels (▲) and collagen sponges (□), respectively (n = 5). In contrast to collagen, PEG gels retain the protein almost completely (~90% after 60 h), most likely as a result of precipitation of the poorly soluble protein. Upon addition of exogenous active MMP-2, gels are degraded and the protein is released (n = 1), mimicking the cell-controlled release of the morphogen that can also occur *in vivo*.

(Fig. 4C). Gel formulations that lacked either rhBMP-2 (Fig. 4A) or MMP sensitivity (Fig. 4B) showed substantially less cell infiltration and bone formation. rhBMP-2 administration in Helistat collagen sponges, used as a positive control, led to bridging of bone that was histologically similar to that in the MMP-sensitive, rhBMP-2–bearing synthetic matrices, except that more bone seemed to be present in the center of the implant in the synthetic matrix (Fig. 4C,D). Whereas considerable amounts of residual collagen remained in the implant at five weeks (Fig. 4D), no residual synthetic matrix was found in any animal (Fig. 4C), demonstrating that complete matrix remodeling can occur without negative consequences to the newly formed bone.

We used microcomputed tomography to quantitatively analyze the cranial samples (Figs. 4E,F and 5). Calculations were made of the volume of bone in the defect, the connectivity of that bone, and the coverage of the defect with new bone by a pseudo-X-ray image obtained by calculating a projected image (in superior-inferior direction) from the 3-D microtomographic data set (Fig. 4E). By measures of bone volume and bone coverage, both MMP sensitivity ($P < 0.03$ for bone volume and $P < 0.02$ for bone coverage) and the presence of rhBMP-2 ($P < 0.01$ for both) were necessary to obtain good healing, similar to that with collagen sponges and rhBMP-2 ($P > 0.5$ for both). Although both histological and microtomographic imaging was qualitatively suggestive of higher connectivity in the bone formed in the MMP-sensitive, rhBMP-2–bearing synthetic matrix than in rhBMP-2–bearing collagen matrices (Fig. 4C,D,F), this trend did not show a high level of statistical significance (P = 0.08; Fig. 4E). Three-dimensional microtomographic projections (Fig. 4F) and two-dimensional serial sections (Fig. 5) were particularly revealing of the morphology of the healed bone.

The influence of cell-triggered matrix degradation on the healing outcome was most obvious when the crosslink density of the materials was changed by forming gels with different PEG macromers, namely 4armPEG-15 kDa (Fig. 6A) as contrasted with the 4armPEG-20 kDa (Fig. 6B) used in the measurements described above (corresponding to a change in volumetric swelling ratio of ~50%). The RGD ligand density and the MMP-sensitive peptide were not changed. Microcomputed tomography demonstrated equivalent coverage of the defect with bone in both cases, but higher bone volume in the 4armPEG-20kD matrix (Fig. 6C,D). In the more densely crosslinked network, bone formation was limited to the

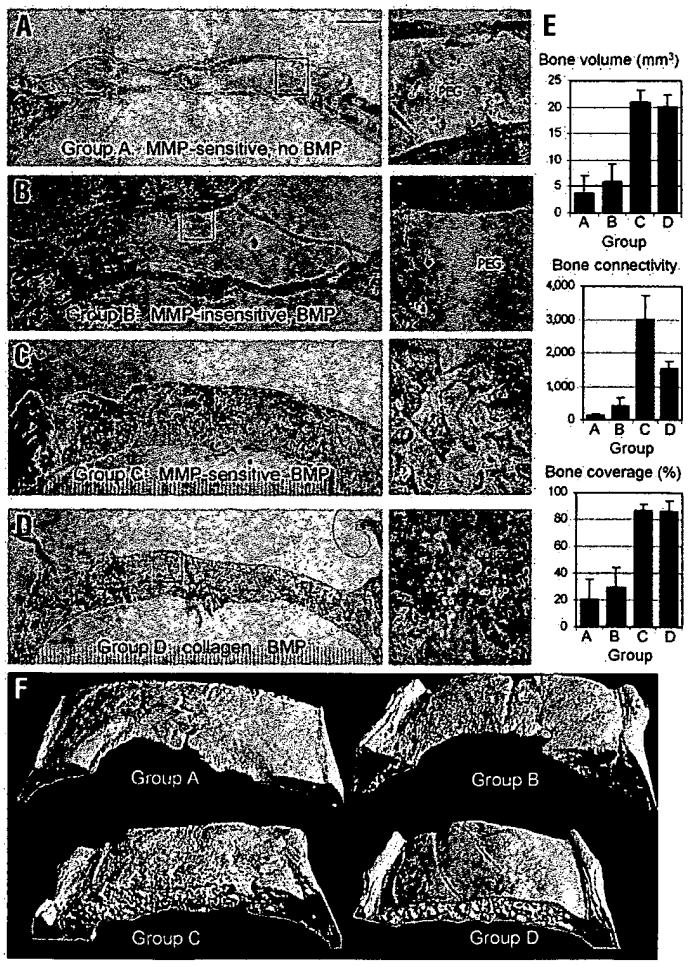

Figure 4. Bone healing in rat calvaria. Healing of critical-size rat calvarial defects five weeks after implantation (n = 5). (A–F) The ability of various matrices to promote healing was assessed by histology (A–D; scale bar, 1 mm) and microcomputed tomography (quantitatively in E, 3-D perspective in F). Quantitative healing indices were derived from microcomputed tomography measurements, namely bone volume (mm^3 of new bone in the defect), bone connectivity (see Experimental Protocol), and bone coverage of the defect (from a pseudoradiograph, reconstructed from the high-resolution microcomputed tomography images). In the synthetic matrices, the presence of rhBMP-2 and the enzymatic sensitivity of the matrix were critical for good healing: gels without physically entrapped rhBMP-2 (A) showed significantly less bone formation ($P < 0.01$ for bone volume, bone connectivity, and bone surface), as did gels that were not susceptible to degradation by proteases (B) ($P < 0.03$ for bone volume, 0.02 for connectivity, 0.02 for bone surface). In both of these specimens, large regions of noninfiltrated matrix (PEG) could still be found. Invasion of cells was observed in some areas (marked with *) of the MMP-sensitive matrix without rhBMP-2. MMP-sensitive and adhesive PEG gels that contained 5 µg rhBMP-2 promoted very good healing of the defects (C). New bone with bony trabeculae and developing marrow was formed. As determined by microcomputed tomography, the healing provided by this synthetic material was not statistically different from the widely used collagen sponges (D), ($P > 0.50$ for bone volume and bone surface). However, the histology and the 3-D renderings from microcomputed tomography are suggestive of higher bone connectivity in specimens treated with the synthetic matrix with rhBMP-2 than with a collagen sponge with rhBMP-2 (P = 0.08). Histological sections of representative samples were selected. Microcomputed tomographic reconstructions of animals with median bone volume were selected.

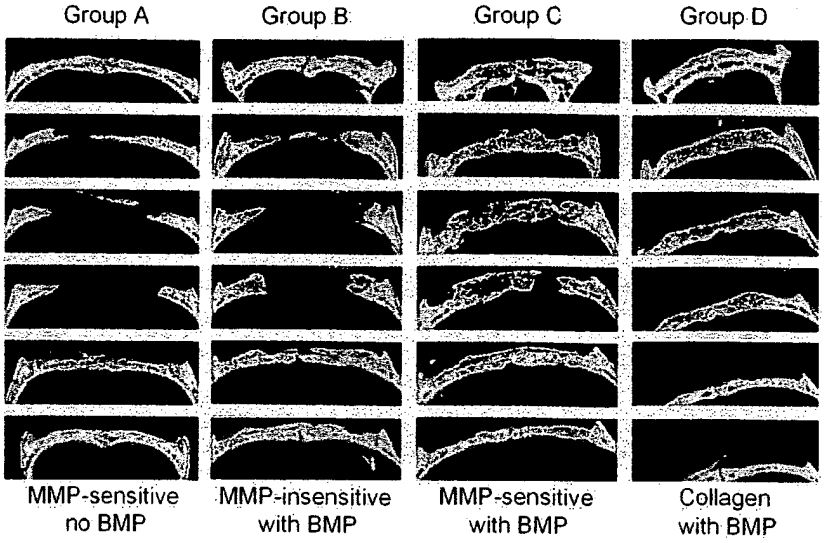

Figure 5. Micro-CT imaging of bone healing. Serial two-dimensional sections of microcomputed tomography of cranial samples of median bone volume. The requirement for both MMP-sensitivity and rhBMP-2 presence (Group C) can be clearly observed (Group A lacks MMP-sensitivity, and Group B lacks rhBMP-2 presence). Animals treated with collagen and rhBMP-2 (Group D) qualitatively demonstrated higher bone density close to the bone-tissue interface than animals treated with the synthetic matrix with rhBMP-2.

material interface with the dura, whereas it penetrated into the depths of the matrix in the 4armPEG-20kD, MMP-sensitive, adhesive matrix in the presence of rhBMP-2. Thus the gel architecture (here, crosslink density) had a profound influence on the speed of cell-triggered enzymatic remodeling.

It was reported that healing of bony tissue with Helistat sponges depends on the pharmacokinetics of BMP release[8]. Helistat is a fibrillar collagen sponge with pores up to several hundreds of micrometers in size. The porosity thus permits rapid cell infiltration without the need for enzymatic degradation. In contrast, our synthetic PEG-based gels require MMP-mediated degradation for cell invasion. We used this activity to locally trigger rhBMP-2 release with the aim of inducing a more localized healing response. This may be especially important in human applications, where larger doses of BMPs are required, and could also be an advantage when BMPs are applied in regions prone to heterotopic ossification, such as the hip or shoulder joints[36]. Indeed, concerns of heterotopic ossification were expressed by the European Agency for the Evaluation of Medicinal Products in its opinion of a product based on BMP-7 delivered in collagen particles derived from bovine bone[37]. Moreover, in the trial considered in this opinion, 10% of patients developed antibodies against BMP-7 and 5% developed antibodies against collagen[5], and this was of significant concern to the regulatory authorities[37]. The immunological benefits of a synthetic matrix compared with a biological one may be substantial. Although it is possible to develop antibodies directed against peptides, it is difficult to do so against peptides as small as the ones we use without conjugating them to larger antigenic species.

The biomimetic matrices described here combine the advantages of synthetic materials and of native protein-based materials such as collagen. Each component of the system can be reproducibly synthesized by chemical means with no risk of disease transmission. The biological efficacy of each component can be independently modulated in a defined way, starting from a passive background (PEG) that is not involved in signaling. The network architecture can be tailored by altering the functionality and molecular weight of PEG macromers. We have demonstrated in this report that such gels are suitable matrices to induce bone regeneration *in situ*. The applied synthesis scheme is rather adaptable, so in principle thiol-containing peptides and proteins of any structure or function can be incorporated. These materials should be useful in many tissue engineering applications and also in more fundamental studies of cell-matrix interactions.

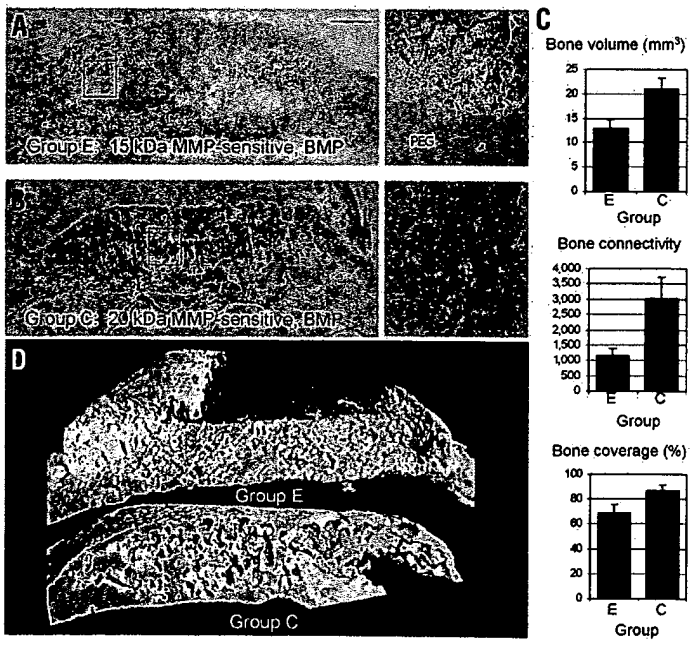

Figure 6. Effect of gel architecture. Healing depended on the hydrogel architecture. (A–D) MMP-sensitive and RGD-functionalized gels composed of 4armPEG-15 kDa (A) and 4armPEG-20 kDa (B) did not show significant differences with regard to bone coverage (C), but morphological differences were clearly apparent from histology and microcomputed tomography (D), which showed that infiltration was much more extensive in the synthetic matrices of lower crosslink density.

Experimental protocol

Materials. Branched PEGs (4arm PEG, 10 kDa; 4arm PEG, 20 kDa) were purchased from Shearwater Polymers (Huntsville, AL). Divinylsulfone was purchased from Aldrich (Buchs, Switzerland). Details of how branched PEG vinylsulfones were produced and characterized are available upon request from authors. All peptides (synthesis chemicals from Novabiochem, Läufelfingen, Switzerland) were synthesized on solid resin using an automated peptide synthesizer (PerSeptive Biosystems, Farmington, MA) with standard F-moc chemistry. Purification was performed by C18 chromatography (Biocad 700E; PerSeptive Biosystems) and peptides were analyzed by matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry. Collagen sponges (Helistat) were purchased from Integra Life Sciences Corporation (Plainsboro, NJ). Helistat is a crosslinked atelopeptide product composed of type I collagen, derived from bovine Achilles tendon.

Formation of PEG-based hydrogels. The synthesis of hydrogels was accomplished through Michael-type addition reaction[38] of thiol-containing peptides onto vinylsulfone-functionalized PEG. A typical adhesive and MMP-sensitive gel of 50 µl volume containing 10% (w/w)

PEG was formed by dissolving 5 mg PEG in 20 µl triethanolamine buffer (0.3 M, pH 8.0) and reacting this solution with 10 µl of 1 mM RGD (Ac-GCGYG*RGDSP*G-NH_2) in a first step. This solution was then mixed with 10 µl of a precursor solution (in the same buffer) containing a peptide with an MMP substrate (Ac-GCRD-GPQGIWGQ-DRCG-NH_2) or an MMP-insensitive control sequence (Ac-GCRD-GDQGIAGF-DRCG-NH_2) flanked by charged amino acids (Arg-Asp) and two Cys residues to render it more soluble and allow formation of a network, respectively.

Cell culture and 3-D cell invasion assay. Human foreskin fibroblasts (neonatal normal human dermal fibroblast; Clonetics, San Diego, CA) were grown in fibroblast cell culture medium (Dulbecco's Modified Eagle's Medium, with 10% fetal bovine serum and 1% antibiotic-antimycotic; Gibco-BRL, Life Technologies, Grand Island, NY) at 37 °C and 5% CO_2. A cell invasion assay was used according to a previously published method[24] in which hFF clusters within fibrin clots were embedded inside 10 µl hydrogel discs by placing a cluster into the precursor solution before gelation. Cell invasion was imaged (inverted phase contrast microscopy, Zeiss Axiovert 135, Zeiss (Feldbach, Switzerland)) and quantified with their center plane in focus (n = 9–12 per group).

rhBMP-2 preparation and release study. rhBMP-2 was prepared as described previously[36]. 5 µg rhBMP-2 was physically entrapped into PEG gels by mixing it with the PEG precursor before gelation. Helistat sponges were loaded with 5 µg rhBMP-2 by absorbing a solution (0.1 mg/ml) into a sponge strip and incubating for 1 h at 37 °C. rhBMP-2 release from both materials was studied based on a previously described method[31]. Samples were placed in 1 ml of TBS (pH 7.4) and stored at 37 °C and 100% humidity. The buffer was replaced every 12 h and refrigerated for further analysis. Finally, four synthetic gels were degraded with MMP-2 (10 nM; Oncogene, Reinbach, Switzerland) at different time points and the amounts of rhBMP-2 in the washes and degraded gels were analyzed using a Bio-Dot SF microfiltration apparatus (Bio-Rad, Hercules, CA) with a slot-blot device. In some experiments, a 25 µm GM6001 (Chemicon, Hofheim, Germany) was added as an MMP inhibitor.

Rat cranial surgery. Eight-millimeter diameter craniotomy defects were created with a trephine in a dental handpiece, carefully avoiding dural perforation. The surgical area was flushed with saline to remove bone debris and a preformed gel was placed within the defect. The soft tissues were closed with skin staples. Rats were killed by CO_2 asphyxiation five weeks after gel implantation. Craniotomy sites with 5 mm contiguous bone were recovered from the skull and placed in 40% ethanol. Twenty-five animals were randomly divided into five groups: 4PEG20-MMP(W)$_X$-RGD + 0 µg rhBMP-2 (Group A), 4PEG20-(DF)$_X$-RGD + 5 µg rhBMP-2 (Group B), 4PEG20-MMP(W)$_X$-RGD + 5 µg rhBMP-2 (Group C), 4PEG15-MMP(W)$_X$-RGD + 5 µg rhBMP-2 (Group E), Helistat (collagen) sponges + 5 µg rhBMP-2 (Group D). Abbreviations: 4PEG15/20 corresponds to the macromers 4armPEG-VS 15 kDa and 20 kDa, respectively. MMP(W)$_X$ corresponds to the MMP-sensitive peptide Ac-GCRD-GPQGIWGQ-DRCG-NH_2 bearing the Trp-containing MMP substrate (GPQGIWGQ), (DF)$_X$ to the MMP-insensitive Ac-GCRD-GDQGIAGF-DRCG-NH_2. RGD corresponds to the peptide Ac-GCGYGRGDSPG-NH_2 with the integrin-binding sequence RGDSP. All animal experiments were evaluated and permitted by the Veterinary Authority of the Canton of Zurich according to Swiss Federal Law Nr. 152/1997.

Microcomputed tomography. Explants were analyzed by microcomputed tomography on a µCT 40 imaging system (Scanco Medical, Bassersdorf, Switzerland) providing an isotropic resolution of 18 µm. A constrained Gaussian filter was used to partly suppress the noise in the volumes. Mineralized bone tissue was segmented from nonmineralized tissue using a global thresholding procedure[39]. All samples were binarized using the same parameters for the filter width (1.2), the filter support (1), and the threshold (224; in permille of maximal image gray value, corresponds to an attenuation coefficient of 1.8 cm^{-1}). Bone connectivity expresses the total number of connections in the bone volume and was directly derived from the Euler number[40]. Bone coverage was calculated from a projection of the cranium in superior-inferior direction to create a high-resolution pseudoradiograph. Three-dimensional visualizations were created using in-house software for surface triangulation and rendering[39,41].

Histology. Samples were prepared for histology[31] and stained with toluidine blue O and Goldner Trichrome (Sigma, Buchs, Switzerland).

Statistics. Statistical analyses of the data were performed using Statview 4.5 (Abacus, Berkeley, CA). Comparative analyses were completed using an unpaired, nonparametric Mann-Whitney test at a 95% confidence level. Mean values and s.d. are shown.

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Competing interests statement

The authors declare competing financial interests: see the Nature Biotechnology website (http://www.nature.com/naturebiotechnology) for details.

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PATENT NO.	:	4,947,840
ISSUED	:	August 14, 1990
INVENTOR(S)	:	Ioannis V. Yannas et al.
PATENT OWNER	:	Massachusetts Institute of Technology
PRODUCT	:	INTEGRA® Artificial Skin

This is to certify that there has been presented to the

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an application under 35 U.S.C. § 156 for an extension of the patent term. Since it appears that the requirements of the law have been met, this certificate extends the term of the patent for the period of

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from August 21, 2007, the original expiration date of the patent, subject to the provisions of 35 U.S.C. § 41(b), with all rights pertaining thereto as provided by 35 U.S.C. § 156(b).

I have caused the seal of the Patent and Trademark Office to be affixed this 23rd day of October 1998.

Bruce A. Lehman
Assistant Secretary of Commerce and
Commissioner of Patents and Trademarks

United States Patent [19]

Yannas et al.

[11] **Patent Number: 4,947,840**

[45] **Date of Patent: Aug. 14, 1990**

[54] **BIODEGRADABLE TEMPLATES FOR THE REGENERATION OF TISSUES**

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[21] Appl. No.: **87,772**

[22] Filed: **Aug. 21, 1987**

[51] **Int. Cl.**[5] **A61L 15/00; A61F 2/10**

[52] **U.S. Cl.** **128/156**; 424/DIG. 13; 623/15; 523/113; 523/114

[58] **Field of Search** 128/156, 897, 898; 623/15; 600/36; 523/114, 113; 424/DIG. 13

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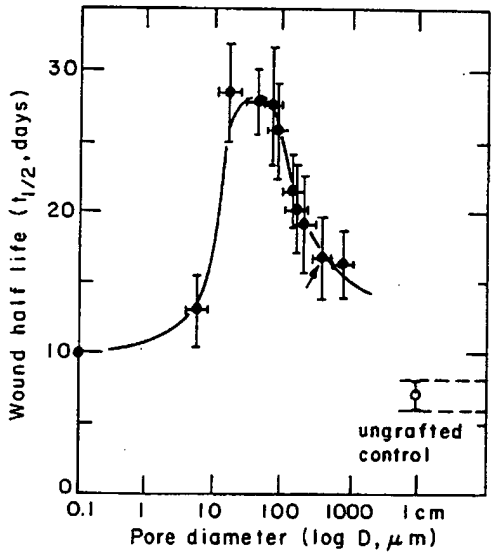
[57] **ABSTRACT**

This invention relates to porous, biodegradable materials in which the pore size, biodegradation rate, and pore volume fraction are controlled and within values at which skin contraction rates around an implant-containing wound are delayed or slowed.

12 Claims, 2 Drawing Sheets



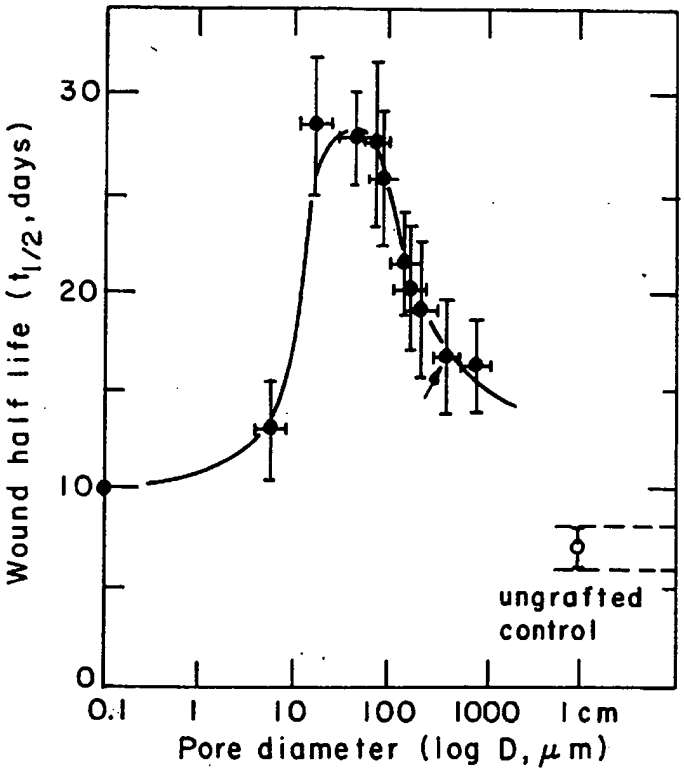

Wound half life ($t_{1/2}$, days)
30
20
10
0
0.1
1
10
100
1000
1 cm
Pore diameter (log D, μm)
ungrafted
control

FIG. 1

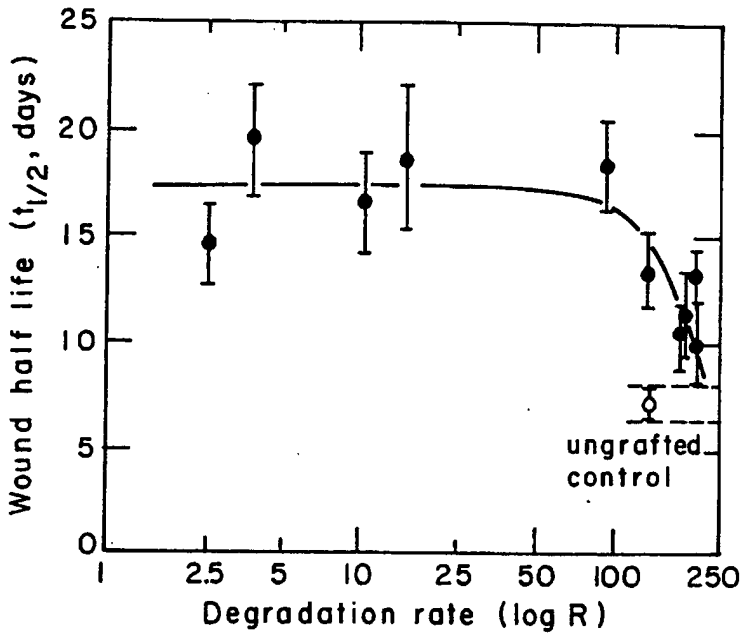

Wound half life ($t_{1/2}$, days)
25
20
15
10
5
0
ungrafted control
1
2.5
5
10
25
50
100
250
Degradation rate (log R)

FIG. 2

BIODEGRADABLE TEMPLATES FOR THE REGENERATION OF TISSUES

Government Support

The invention described herein was supported in whole or in part by a grant from the National Institutes of Health.

Background of the Invention

Currently there is a variety of general approaches to the treatment of loss of tissue which results either from trauma or from elective surgery. One approach, transplantation, suffers from the fact that the host typically rejects the tissue of the donor. A second, autografting, bypasses the problem of immunological rejection but suffers from two disadvantages: tissue for autografting must be removed from the patient thereby subjecting the latter to additional serious surgery and, additionally, when the trauma is massive there is not sufficient intact tissue to be harvested for the autografting. A third approach attempts to synthesize tissues by culturing in vitro cells of the patient and, suffers from the fact that the culture process is lengthy as well as from the fact that techniques are not still generally available for culturing tissues (the epidermis seems to be the only exception). A fourth approach to tissue repair consists in the fabrication of devices using metals (hip prosthesis), ceramics (bone prosthesis) or polymers (artificial heart). This approach suffers from the acute incompatibility, (mechanical, physicochemical and biological), between implant and host tissue which typically leads to device failure within a period of time which is much less that the patient's lifetime.

A fifth approach to treatment of tissue loss consists in treating the wounded tissue with a biodegradable polymeric material which, acting as a scaffold, induces the wound to synthesize new tissue.

An example of such a biodegradable polymeric material is a material formed from crosslinked collagen molecules that are covalently bonded to glycosaminoglycan molecules. These polymer materials have been described in the scientific literature and the patent literature. See, for example, U.S. Pat. No. 4,505,266 (Mar. 19, 1985); U.S. Pat. No. 4,448,718 (May 15, 1984); U.S. Pat. No. 4,418,691 (Dec. 6, 1983); U.S. Pat. No. 4,458,678 (July 10, 1984); U.S. Pat. No. 4,350,629 (Sept. 21, 1982); U.S. Pat. No. 4,522,753 (June 11, 1985); U.S. Pat. No. 4,280,954 (July 28, 1981); U.S. Pat. No. 4,252,759 (Feb. 24, 1981); and U.S. Pat. No. 4,060,081 (Nov. 29, 1977) the teachings of which are incorporated herein by reference.

A need has long existed, however, for an artificial skin implant in which the rate of skin contraction around a wound is lowered, thereby reducing the amount of scar tissue formed.

Summary of the Invention

The present invention is based upon the discovery that the contraction rate of full thickness skin wounds around an artificial skin implant may be significantly slowed if the biodegradation rate and average pore size of the implant are maintained within selected parameters. Specifically, this invention pertains to a polymeric, biodegradable material in which both the biodegradation rate and the pore size are selected and maintained to provide efficacious use of the material as a scaffold for wound repair. One specific example of a material is a crosslinked collagen-glycosaminoglycan in which crosslinking has been carried out by contacting the composite with an aqueous glutaraldehyde solution for a sufficient time. This yields a degree of crosslinking which is inversely proportional to the rate of biodegradation of the material. Additionally, the material is produced in a manner in which the average pore size is between approximately 9 μm and approximately 630 μm.

Brief Description of the Figures

FIG. 1 is a plot correlating the effects of average pore size of a crosslinked collagenglycosaminoglycan skin graft implanted at the wound site of guinea pigs on the inverse rate of wound closure by contraction.

FIG. 2 correlates the dependence of the rate of wound closure on degradation rate for collagen-GAG grafts which were crosslinked in aqueous glutaraldehyde.

Detailed Description of the Invention

The key to a successful artificial skin implant lies in certain critical parameters of the implant material. These parameters are the biodegradation rate of the material, the mean pore size of the material, and the pore volume fraction of the material.

Artificial skin implants may be produced from a variety of collagen-based materials in which the collagen, either alone or in combination with a glycosaminoglycan, a glycoprotein, a structural protein or a growth factor is crosslinked. Suitable glyoproteins include fibronectin, laminin and chondronectin. A suitable structural protein is elastin. Growth factors may be epidermal growth factor, platelet derived growth factor, tissue angiogenesis factor, nerve growth factor and bone growth factor. In addition, certain collagen-free materials can also be used, provided they have the proper combination of critical parameters discussed above. For example, synthetic materials such as polylactides or polyglycolic acids, can be used as skin implants.

Methods of preparing polyactides are well documented in the patent literature. The following U.S. Patent Nos., the teachings of which are hereby incorporated by reference, describe in detail suitable polylactides, their properties and their preparation: Dorough, U.S. Pat. No. 1,995,970; Schneider, U.S. Pat. No. 2,703,316; Salzberg, U.S. Pat. No.2,758,987, Zeile, U.S. Pat. No. 2,951,828; Higging, U.S. Pat. Nos. 2676,945; 2,683,136; Trehu, U.S. Pat. No. 3,531,561; British Patent specification Nos. 755,447; 779,291; 825,335, 901,037; 932,382; 1,048,088; 1,123,445; West German Patent Nos. 946,664; 975,191; 1,112,293; 1,152,258; 1,153,902; East German Patent Nos. 14,548; French Patent Nos. 1,425,333; 1,478,694; 1,512,182; Netherlands Patent Nos. 99,836; 6,605,197; 6,605,292; Japanese Patent Nos. 17,675 (1966); 7,796 (1967); 2,948 (1968); 15,789 (1969).

Polyglycolic acids and their properties are described in more detail in the following article, the teachings of which are hereby incorporated by reference: "Cyanamid Research Develops World's First Synthetic Absorbably Suture", Chemistry and Industry, July 11, 1970, Page 905.

A preferred artificial skin implant comprises a composite formed from collagen molecules that are crosslinked and covalently bonded with glycosaminoglycan (GAG). Examples of specific glycosaminoglycans are chondroitin 6-sulfate, chondroitin 4-sulfate, heparan,

heparan sulfate, keratan sulfate, dermatan sulfate, chitin or chitosan. Such a composite can be made by forming an uncrosslinked material comprising a reaction product of collagen and a glycosaminoglycan and contacting the reaction product with an aqueous glutaraldehyde solution for a period in excess of one hour. The resulting crosslinked collagen-glycosaminoglycan composite has a rate of biodegradation which is low enough to enable the composite to be a suitable scaffold for wound repair.

The biodegradation rate of the crosslinked implant material is inversely related to the degree of crosslinking in the material. It has been found that crosslinked composites should have an average molecular weight between crosslinks, (M_c), of between about 800 and about 60,000 daltons. Materials with M_c values below about 800 and above about 60,000 suffer significant losses in their mechanical properties. Composites with an M_c of between 10,000 and about 40,000 tend to have the best balance between physical and therapeutic properties. Thus, this is the preferred range of crosslinking for products requiring such a balance of properties.

Covalent crosslinking can be achieved by many specific techniques with the general catagories being chemical, radiation and dehydrothermal methods. An advantage to most crosslinking methods contemplated, including glutaraldehyde crosslinking and dehydrothermal crosslinking, is that they also serve to remove bacterial growths from the materials. Thus, the composites become sterilized while simultaneously being crosslinked.

One suitable method for covalently crosslinking the collagen-GAG composites is known as aldehyde crosslinking. In this process, the materials are contacted with aqueous solutions of aldehyde, which serve to crosslink the materials. Suitable materials include formaldehyde, glutaraldehyde and glyoxal. Glutaraldehyde is preferred because it yields the desired level of crosslink density more rapidly than other aldehydes and is also capable of increasing the crosslink density to a relatively high level. It has been noted that immersing the composites in aldehyde solutions causes partial removal of the polysaccharide component by dissolution thereby lessening the amount of polysaccharide in the final product. Unreacted aldehydes should be removed from the collagen-GAG material by exhaustive rinsing with water since residual aldehydes are quite toxic.

Other suitable chemical crosslinking techniques include carbodiimide coupling, azide coupling and diisocyanate crosslinking.

Another crosslinking method is referred to herein as a dehydrothermal process. In dehydrothermal crosslinking, no external crosslinking agents need to be added to the composite. Rather, the composite is dehydrated to a moisture content of less than about 1%. The actual amount of water which must be removed will vary with many factors, but, in general, sufficient water to achieve the desired crosslinking density must be removed. Thus, a collagen-GAG product can be subjected to elevated temperatures and/or vacuum conditions until the moisture content is reduced to extremely low levels and the desired crosslinking density is achieved. In the absence of a vacuum, temperatures about 80° C., and perferably above about 90° C. can be used. On the other hand, if the process is to be performed at approximately 23° C., a vacuum of at least 10^{-5} mm Hg, and preferably below about 10^{-6} mm Hg is suitable. In the preferred embodiment of this process, elevated temperatures and vacuum can be used in combination to expedite crosslinking. With a vacuum of at least about 10^{-5} mmHg, it is preferred to use a temperature of at least about 35° C. In general, the materials are subjected to the elevated temperatures and vacuum conditions until the desired degree of crosslinking density is achieved. The higher the temperature, the lower is the vacuum required to arrive at a given crosslink density; and vice versa. This dehydrothermal crosslinking process overcomes certain disadvantages of the aldehyde crosslinking method and produces composites having relatively large amounts of GAG strongly bound to the collagen chain.

The exact mechanism operating in the dehydrothermal crosslinking process in not known. However, it is believed to be either an amide condensation involving ϵ-amino groups from collagen and carboxyl groups from the GAG component, or esterification involving carboxyl groups from the GAG component and hydroxyl groups from the collagen, or esterification involving hydroxyl groups from the GAG component and carboxyl groups from the collagen. It is possible all three mechanisms are involved to some extent. For a more detailed description of dehydrothermal crosslinking, see Yannas, I.V. and Tobolsky, A.V., "Crosslinking of Gelatin by Dehydration", Nature, vol. 215, # 5100, pages 509–510, July 29, 1967, the teachings of which are hereby incorporated by reference.

The degree of crosslink density is an important parameter of this invention since it is a direct, controlling factor in the degradation rate of the material. Generally, the greater the crosslink density, the lower the degradation rate, and vice versa. Thus, by controlling the degree of crosslinking, it is possible to produce composites which exhibit a degradation rate within a range determined to be clinically desirable. The maximum degradation rate has been determined to be about 140 enzyme units (e.u.) as measured in a test described below. This upper limit in degradation rate is critical since it has been determined that implants with degradation rates higher than about 140 e.u. fail to significantly delay wound contraction. In the preferred embodiment of this invention, the biodegradation rate is below about 120 e.u.

The effectiveness of polymeric scaffolds in slowing or delaying contraction around a wound is further increased by controlling the average pore size of the implant within an upper and a lower limit. To effectively slow or delay wound contraction, and thus serve the purpose of this invention, the implant must contain pores whose average size is within the range of about 9 μm to about 630 μm. In the preferred embodiment of this invention, the average pore size is within the range of about 9 μm to about 630 μm. Both the 9 μm and the 630 μm limits are critical as it has been found that implants with average pore sizes smaller than about 9 μm or greater than about 630 μm fail to significantly delay wound contraction.

Polymeric materials which are fabricated by methods which yield a low enough biodegradation rate and pore size within the desired upper and lower limits have been found to effectively delay or arrest skin wound contraction and induce synthesis of new functional tissue. Materials which do not come within the proper parameters do not delay or arrest skin wound contraction and tend to induce synthesis of undesirable scar tissue.

Another determining factor in the effectiveness of artificial skin implants is the pore volume fraction of the

implant material. This value is defined as the percentage of the total volume of the material which is occupied by pore space. A more detailed definition is given in Fischmeister, H.F., Proceedings Int. Symp. RILEM/I-UPAC, Prague, Sept. 18–21, 1973, Final Report Part II, p. C-439 the teachings of which are incorporated herein by reference. A high pore volume fraction has been found to be clinically desirable, with implants having pore volume fractions above about 80% being preferred.

Exemplification

Bovine hide collagen, chondroitin-6-sulfate (C-6-S, 0.11% w/v), acetic acid (0.05M, pH 3), deionized water, medical-grade 7-mil silicone sheeting and silicone medical adhesive, isopropanol (70%), and phosphate-buffered saline, were used in the manufacture and processing of the collagen-GAG membranes. The crosslinking agent was glutaraldehyde (reagent grade, Aldrich Chem. Col, Milwaukee, WI) diluted in 0.05M acetic acid.

Membranes for use as controls were prepared according to the Stage 1 artificial skin protocol developed by Yannas et al. "Design of an Artificial Skin", *J. Biomed. Mat. Res.*, 14:65–81, 107–131, 511–528 (1980). The specific procedure employed is now set forth and unless otherwise noted, the procedures were done at room temperature.

1. Blend 1.65 g of milled collagen with 600 ml of 0.05 M acetic acid (pH3) for 1 hour at 4° C.
2. Dropwise add 120 ml of C-6-S solution to the blending collagen dispersion over 15 minutes at 4° C. (8% w/w of C-6-S is added to the collagen, but no assay was done to measure the amount retained after the last step.)
3. Blend an additional 15 minutes at 4° C.
4. Centrifuge at 1500g in a 4° C. centrifuge for 105 minutes.
5. Decant 420 ml of supernatant.
6. Reblend slurry for 15 minutes at 4° C.
7. Pour into stainless-steel trays (2 ml per square inch of tray surface); freeze 1 hour at −40° C.
8. Allow to lyophilize (freeze-dry) 24 hours at 0° C. and 100 mtorr.
9. Place foam in vacuum oven at 105° C., 50 mtorr for 24 hours (dehydrothermal treatment).
10. Seal and store in desiccator until ready for next step.
11. Apply silicone layer.
12. Rehydrate in 0.05 M acetic acid (pH 3) for 24 hours.
13. Crosslink in 0.25% glutaraldehyde (pH 3) for 24 hours.
14. Rinse thoroughly with deionized water.
15. Immerse in deionized water for 24 hours.
16. Store in 70% isopropanol at 4° C.

Steps 10 through 16 were done with sterile technique if the artificial skin was to be used for animal experimentation. Step 11 was omitted for membranes that were not to be used in vivo. For membranes to be used as grafts and also to be tested in vitro, the silicone was applied to only about a third of the membrane. Membranes containing different amounts of GAG were prepared by using different concentrations of C-6-S for Step 2 discussed above. For membranes containing no GAG (i.e., crosslinked collagen) Step 4 was changed to centrifugation at 17,000g at 4.C for 2 hours. This was necessary due to the reduction in density of the collagen dispersion caused by the absence of GAG.

One method of varying crosslink density was to vary the length of crosslinking time in 0.25% glutaraldehyde (Step 13). Another way was to multiply the concentration of glutaraldehyde ten times to 2.5% while keeping the crosslinking time at 24 hours.

A third, completely different method of inducing very high crosslink densities was crosslinking with glutaraldehyde vapor at controlled temperature and relative humidity.

To determine the approximate average pore size in the collagen-GAG membranes, samples were embedded in methacrylate, sectioned to 5 μm thickness, mounted on a glass slide, and stained with 1% toluidine blue. The slides were examined under a light microscope at 125x magnification. The diameter of the field of vision through the microscope was 0.138 inch. The number of pores across the field was counted at various locations on the slide and different orientations. At least 6 such countings were done for each membrane. Pore size was calculated at the field size divided by the number of pores across the field. The mean and standard deviation of the 6 or more numbers representing pore size were then calculated.

The molecular weight between crosslinks in the membranes, M_c, was determined by the M_c test. In this test, strips of artificial skin material were gelatinized in 80° C. normal saline and the equilibrium tensile stress was studied as a function of equilibrium strain. Gelatinization destroys the triple helical structure of collagen. The gelatinized material is modelled as a swollen, ideal rubber. The M_c can be calculated from the equilibrium stress-strain relation of the material. For each type of membrane, at least 4 strips were tested. The mean and standard deviation of the M_c values of these strips were calculated. Molecular weight between crosslinks is an inverse measure of crosslink density. A more detailed discussion of the M_c test may be found in U.S. Pat. No. 4,060,081, the teachings of which have previously been incorporated by reference.

The biodegradation rate of the various membranes was characterized using a modified collagenase assay from the Sigma Chemical Company (1977). In preparation for the assay, the membranes were first refreeze-dried (Steps 7 and 8 discussed previously).

In the assay, the material to be tested was incubated for 5 hours with collagenase from Clostridium bistolyticum (GIBCO, Grand Island, N.Y.). This bacterial collagenase hydrolyzes proteins containing proline. The amino groups liberated were measured as equivalents of L-leucine using a colorimetric ninhydrin method. The darker the final solution, the higher the concentration of free amino groups, and thus the faster the degradation of the material.

Bovine Achilles tendon collagen (Type I, insoluble) from the Sigma Chemical Company was used as a standard. It gives a known activity of 202 enzyme units. By definition (Sigma 1977), one enzyme unit "will liberate Amino Acids from Collagen equivalent in Ninhydrin color to 1.0 Mole of L-Leucine in 5 hours at pH 7.4 at 37° C."

The units measured using this assay are referred to as "enzyme units". Enzyme units are a measure of the in vitro degradation rate of the material being tested. The higher the number of enzyme units, the greater the degradation rate.

Unprocessed 20-mesh milled bovine collagen (the major raw material in the manufacture artificial skin) was used as a control. When the enzyme units of a day's assay were calculated and normalized to the Sigma standard of 202, the bovine collagen was expected to yield enzyme units in the range of 178±15.

The following summarizes the steps in the collagenase assay.

1. For each collagen type weigh out 0.025 g of collagen into each of 4 test tubes; one tube is the spectrophotometric blank.
2. Incubate each test sample in 5 ml of a pH 7.4 buffer with 0.10 ml of a buffered solution of 0.08% w/v collagenase (0.10 ml deionized water in each blank) for 5 hr at 37° C.; stir continuously by using a magnetic flea in each tube.
3. Filter the contents of each tube to eliminate turbidity that may interfere with the spectrophotometric measurements; save 0.20 ml of each filtrate.
4. Add 2 ml of a pH 5.5 ninhydrin-and-hydrindantin solution to stop the enzymatic reaction and to induce color.
5. Place the tubes in a boiling water bath for 20 min.
6. Mix in 10 ml of 50% propanol, or 5 ml for highly crosslinked materials; let stand 15 min at room temperature to develop and fix color.
7. Record transmittance at 600 nm.

Steps 4 through 7 were also done on a calibration tube containing 0.20 ml of 0.002M L-leucine and on a corresponding blank containing 0.20 ml of deionized water.

The enzyme units of each test (3 test tubes per collagen type) were calculated from the transmittances and by comparison to the calibration test indicating the transmittance of a known amount of L-Leucine. The mean standard deviation of each set of 3 results was determined. Finally, these numbers were multiplied by the factor which normalized the mean enzyme units for Sigma collagen to 202.

A bacterial collagenase is used in the assay, while the collagenase in a guinea pig or human is mammalian. This means that the enzyme units do not indicate the actual degradation rate of a graft in a wound. Rather, the assay is an in vitro tool used to determine quantitatively the relative biodegradabilities of graft materials.

In vivo response to various collagen membranes was studied by grafting onto guinea pigs. White female Hartley guinea pigs (Elm Hill Breeding Labs, Chelmsford, MA) were allowed to stabilize in the MIT Animal Facility for at least one week after arrival at the facility. Each guinea pig weighed between 430 and 810 g at surgery.

Each animal was initially anesthetized by inhalation of 2% Halothane in 0.5 liters/min oxygen and 2 liters/-min nitrous oxide. During the course of surgery, the Halothane level was gradually reduced. Approximately 2 minutes before the completion of surgery, the animal was taken off anesthesia and given 2 liters/min pure oxygen for about 5 minutes.

After the outline of the wound was made with a scalpel on the back of the animal, the dimensions of the outline were measured. Care was taken to avoid any stretching or buckling of the skin at the wound site during measurement. The nominal wound dimensions were 1.5 cm by 3.0 cm. The "original wound area" was calculated as the product of the mean of the long dimensions and the mean of the short dimensions.

Every 3 or 4 days, the area of the wound was determined. Five dimensions were measured: the length at both sides of the wound, the width at both ends, and the width at the middle. Local effects of any sutures pulling the intact skin over the wound were avoided by taking measurements slightly away from such irregularities. In, cases where the wound was far from rectangular, a judgment was made to decide where to make measurements. The intent was to have the product of the means of the length and width measurements most accurately represent the actual area of the wound. Errors in area measurement from human judgment and from stretching or gathering of skin are estimated to be 15%.

The area measured was initially the pink area under the graft. When a slightly darker band appeared around the pink area during the course of healing, this band was also included in the area measured. The pink area eventually disappeared as the wound edges came together, and the darker area remained as a scar. Measurements taken in this way were designated "pink-and-dark area". The forked ends of the scar were not included in the area measurements.

It is believed that the darker area is vascularized collagen-GAG covered by epidermal ingrowth from the wound edges. This area is surrounded by healthy dermis containing hair follicles and hair. The hair closest to the wound edge is often too small to see. Thus, the area within the visible hairline(designated the "hairline area") is an upper bound for the pink-and-dark area.

The main parameter to gauge graft efficacy is a determination of the day at which, following surgery, the wound has reached approximately half of its original area. This parameter is defined as D_{50}. D_{50} roughly indicates the rate of wound closure by contraction. The wound area closes by two mechanisms: epidermal ingrowth from the wound edges (epithelialization), and drawing together of the wound edges (contraction). Epithelialization occurs at a fairly constant rate, whereas contraction is a function of graft material. The greater the D_{50} the slower the contraction, and therefore the better the graft.

Results

Effects of pore size are summarized in FIG. 1. Materials with different pore sizes were prepared in different ways, but all were processed identically after the freezing step (after Step 8 described previously). All were crosslinked in 0.25% glutaraldehyde in 0.05 M acetic acid for 24 hours at room temperature.

Control of the mean pore size was a very effective procedure for delaying wound contraction. Using an arbitrary cut-off level of $D_{50}=15$ days, FIG. 1 may be used to estimate a lower mean pore size limit of about 9 μm and an upper mean pore size limit of about 630 μm. In the preferred embodiment of this invention, a maximum increase of $D_{50}=27$ days occurred when the mean pore size varied between the narrower limits of about 20 μm and about 125 μm.

Thus, it is important to consider both upper and lower limits of pore diameter when comparing the wound-healing efficacy of different graft materials.

Pores are formed by ice nucleation during the freezing step. Pore size is temperature-dependent. At very low temperatures, the collagen-GAG slurry freezes very quickly, locking tiny ice crystals into place soon after nucleation. At higher temperatures (but still below freezing temperatures), the ice crystals have time to

grow and fuse together before the slurry is frozen solid, resulting in larger pores.

It has been shown that the specific surface of the material being tested does not affect the results of the collagenase assay. This implies that differences in pore size do not affect assay values because of any differences in surface area. This is expected since the enzyme attacks the protein molecules on a scale much smaller than the size of the pores formed in the artificial skin during the freeze-drying step.

FIG. 2 shows D_{50} as a function of enzyme units for the grafts which were crosslinked with aqueous glutaraldehydes. To examine the lower end of the range more easily, the degradation rate is plotted on a logarithmic scale. The x-axis standard deviations have been omitted.

The ten fold difference in degradation rate between materials at 20 enzyme units ($D_{50}=17$ days) and those at 200 units ($D_{50}=11$ days) clearly resulted in difference in wound contraction. Slower graft degradation slowed contraction.

It has further been found that D_{50} varies as a function of crosslink density for aqueously crosslinked grafts.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiment of the invention described herein. For example, the glutaraldehyde crosslinking process should not be limited to methods using aqueous glutaraldehyde, as gaseous forms may be used as well. Also, the materials described herein should not be limited to skin implants, but may be used for other organs such as nerve fibers, peridontal tissues and blood vessels. This disclosure is intended to extend to all biodegradable implant materials of the type described in which the degradation rate, pore size and pore volume fraction are within the limits described to yield effective results. Such equivalents are intended to be encompassed in the following claims.

We claim:

1. A method of delaying or arresting contraction of skin bordering a wound site and promoting tissue regeneration, comprising applying a biodegradable material with a pore size of between about 9 μm and 630 μm, a pore volume fraction of greater than about 80%, and a biodegradation rate sufficient to significantly delay or arrest the rate of wound contraction such that the time it takes a wound to contract to one-half of its original area is greater than approximately 15 days.

2. A method as in claim 1 wherein the biodegradable material comprises collagen molecules.

3. A method as in claim 1 wherein the biodegradable material comprises collagen molecules that are crosslinked and covalently bonded with a second material selected from the group consisting of glycosaminoglycans, glycoproteins, structural proteins and growth factors.

4. A method as in claim 3 wherein the second material is a glycosaminoglycan selected from the group consisting of chondroitin 6-sulfate, chondroitin 4-sulfate, heparin, heparan sulfate, keratan sulfate, dermatan sulfate, chitin and chitosan.

5. A method as in claim 3 wherein the second material is a glycoprotein selected from the group consisting of fibronectin, laminin and chondronectin.

6. A method as in claim 3 wherein the second material is a structural protein comprising elastin.

7. A method as in claim 3 wherein the second material is a growth factor selected from the group consisting of epidermal growth factor, platelet derived growth factor, tissue angiogenesis growth factor and bone growth factor.

8. A method as in claim 3 wherein the biodegradable material is crosslinked by contacting it with an aqueous aldehyde.

9. A method as in claim 8 wherein the aldehyde is glutaraldehyde.

10. A method as in claim 1 wherein the biodegradable material contains pores with an average size ranging from about 20 μm to about 125 μm.

11. A method as in claim 1 wherein the biodegradable material has a degradation rate in an in vitro collagenase assay of below about 140 enzyme units.

12. A method as in claim 11 wherein the biodegradable material has a degradation rate in an in vitro collagenase-assay of below about 120 enzyme units.

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